UNIVERSITY
LIBRARY
22 MAR 1960
READING

JOURNAL OF EXPERIMENTAL BOTANY

AN OFFICIAL ORGAN OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY

Volume 10 *October* 1959 *Number* 30

CONTENTS

D. B. IDLE: Studies in Extension Growth, III 337

CHARLOTTE J. AVERS and R. B. GRIMM: Comparative Enzyme Differentiation in Grass Roots, II 341

R. G. HURD: An Effect of pH and Bicarbonate on Salt Accumulation by Disks of Storage Tissue 345

S. LAVEE: The Effect of Some Growth-regulating Substances on the Development of *Sclerotium rolfsii* (Sacc.) 359

R. F. M. VAN STEVENINCK: Factors Affecting the Abscission of Reproductive Organs in Yellow Lupins (*Lupinus luteus* L.), III 367

M. B. WILKINS: An Endogenous Rhythm in the Rate of Carbon Dioxide Output of Bryophyllum, I 377

D. DAVIDSON: Changes in the Chromosome Complements of Cells of *Vicia faba* Roots following Irradiation 391

I. K. VASIL: Cultivation of Excised Anthers *in vitro*—Effect of Nucleic Acids 399

S. P. SPRAGG and E. W. YEMM: Respiratory Mechanisms and the Changes of Glutathione and Ascorbic Acid in Germinating Peas 409

T. F. NEALES: The Boron Requirement of Flax Roots Grown in Sterile Culture 426

L. A. GRIFFITHS: On the Distribution of Gentisic Acid in Green Plants . 437

K. H. SCHÜTTE: Micro-Nutrient Deficiency and Transpiration in *Tropaeolum majus* L. 443

I. MANTON: Observations on the Internal Structure of the Spermatozoid of *Dictyota* 448

N. P. KEFFORD: Some Growth Regulators of Tobacco in Relation to the Symptoms of the Physiological Disease 'Frenching' 462

T. A. BENNET-CLARK and A. W. WHEELER: The Metabolism of 3-Indolylacetic Acid in Plant Tissues. 468

G. E. BLACKMAN and J. A. SARGENT: The Uptake of Growth Substances, II 480

I. D. J. PHILLIPS and P. F. WAREING: Studies in Dormancy of Sycamore, II 504

OXFORD: AT THE CLARENDON PRESS

JOURNAL OF
EXPERIMENTAL BOTANY

Editorial Committee: T. A. BENNET-CLARK (Editor), E. ASHBY, G. E. BLACKMAN, R. BROWN, F. G. GREGORY, W. O. JAMES, W. H. PEARSALL, R. D. PRESTON, M. THOMAS.

THE JOURNAL OF EXPERIMENTAL BOTANY is sponsored by the Society for Experimental Biology as a medium for the publication of papers in the field of plant physiology, biochemistry, biophysics, experimental agronomy, and related topics.
Three numbers comprise a volume and they appear at four-monthly intervals. Subscription price 55*s.* per volume. Single numbers 22*s.* net.

NOTICE TO CONTRIBUTORS

Papers for publication after January 1960 should be addressed to the new editor, Professor W. T. Williams. The right of publication in this journal is not restricted to members of the Society and contributions from research workers both in Great Britain and abroad will be welcomed.

Manuscripts must be typewritten with double spacing. Standard botanical and chemical terminology and abbreviations should be used and contributors are asked to cite references in standard form and present the summary at the beginning rather than at the end of their papers.

Contributors will receive one proof in page. An allowance of 10*s.* per sheet of sixteen pages is made for alterations in the proof, printers' errors excepted, and contributors will be responsible for any excess.

Contributors receive twenty-five copies of offprints free of charge, and may purchase further copies at rates which will be quoted on application to the publisher.

ILLUSTRATIONS

All figures, both text-figures and plates, must be numbered in a single series (figs. 1, 2, 3, &c.). There must not be one numbered series of text-figures and another separately numbered series of plates.

No figure should be sent that will not fit easily into an envelope 40 × 30 cm (about 16 × 12 inches).

The lettering of all figures should be clearly written on tracing paper bearing accurate outlines of the objects or graphs indicated by the lettering.

A typewritten (double-spaced) list of the legends of all figures must be provided. Each legend must be complete in itself. Remarks applying to more than one figure will not be printed.

Text figures and graphs. These must be drawn in black process ink with graphs preferably on quarto size mm graph paper.

Plates. When several photomicrographs are grouped to form a single plate, they should, if possible, be arranged to occupy an area of approximately the right shape to fill the available space (19 × 12 cm), and they must be neatly pasted to a single piece of white cardboard. The photomicrographs in a single plate must be labelled A, B, C, &c. Room should be left to include the legend below the photomicrographs on the plate rather than in a separate list of plates.

[*continued on p. iii of cover*

Studies in Extension Growth

III. A RECORDING AUXANOMETER FOR PROLONGED OPERATION AT A RESOLUTION OF TEN MINUTES AND FIVE MICRONS

D. B. IDLE

Department of Botany, University of Birmingham

Received 17 August 1958

SUMMARY

An auxanometer is described that has been specially designed to study the timing of fluctuations in the extension rate of two separate tissues. It is capable of recording the extension rate on paper strip for long periods, while the tissues add up to 15 cm. to their initial length.

INTRODUCTION

THE auxanometer described previously (Idle, 1955) could record the extension rate of an organ; it was some disadvantage that only 0·5 cm. of growth could be followed without resetting the machine, since the effects on the plant of that operation lasted for a considerable proportion of the total time span available, which in practice was about 10 to 15 hours.

This paper describes an apparatus that has been designed for the study of correlated phenomena in the extension rates of two tissues. The resolution is 10 minutes and 5 μ, over a total distance of 15 cm. Two identical auxanometers are synchronized from a common clock, so that the time relation between events in both tissues may be accurately determined.

The apparatus has been used to study the behaviour of etiolated hypocotyls of *Helianthus annuus*. A specimen result is given to illustrate the abilities of the machine.

THE AUXANOMETER

The operation of this machine is best understood by considering first how the part on the left of the diagram in Fig. 1 follows the growth of the plant, and then how it is that the recording part on the right successfully amplifies the movements of the former.

The machine is really a contact auxanometer in which the switch S is moved upwards as long as it is closed by upward pressure from the plant under observation. The motor winds the switch up on steel wire over pulleys. The wire is held in tension by springs which are not shown in the diagram, but which are inserted in the wire strands as they pass down behind the switch. The pulleys are 1 in. diameter and have two shallow grooves cut to take the wires, which are about $\frac{1}{2}$ in. apart. The switch S operates the relay, R_1,

JOURNAL OF
EXPERIMENTAL BOTANY

Editorial Committee: T. A. BENNET-CLARK (Editor), E. ASHBY, G. E. BLACKMAN, R. BROWN, F. G. GREGORY, W. O. JAMES, W. H. PEARSALL, R. D. PRESTON, M. THOMAS.

THE JOURNAL OF EXPERIMENTAL BOTANY is sponsored by the Society for Experimental Biology as a medium for the publication of papers in the field of plant physiology, biochemistry, biophysics, experimental agronomy, and related topics.

Three numbers comprise a volume and they appear at four-monthly intervals. Subscription price 55*s.* per volume. Single numbers 22*s.* net.

NOTICE TO CONTRIBUTORS

Papers for publication after January 1960 should be addressed to the new editor, Professor W. T. Williams. The right of publication in this journal is not restricted to members of the Society and contributions from research workers both in Great Britain and abroad will be welcomed.

Manuscripts must be typewritten with double spacing. Standard botanical and chemical terminology and abbreviations should be used and contributors are asked to cite references in standard form and present the summary at the beginning rather than at the end of their papers.

Contributors will receive one proof in page. An allowance of 10*s.* per sheet of sixteen pages is made for alterations in the proof, printers' errors excepted, and contributors will be responsible for any excess.

Contributors receive twenty-five copies of offprints free of charge, and may purchase further copies at rates which will be quoted on application to the publisher.

ILLUSTRATIONS

All figures, both text-figures and plates, must be numbered in a single series (figs. 1, 2, 3, &c.). There must not be one numbered series of text-figures and another separately numbered series of plates.

No figure should be sent that will not fit easily into an envelope 40 × 30 cm (about 16 × 12 inches).

The lettering of all figures should be clearly written on tracing paper bearing accurate outlines of the objects or graphs indicated by the lettering.

A typewritten (double-spaced) list of the legends of all figures must be provided. Each legend must be complete in itself. Remarks applying to more than one figure will not be printed.

Text figures and graphs. These must be drawn in black process ink with graphs preferably on quarto size mm graph paper.

Plates. When several photomicrographs are grouped to form a single plate, they should, if possible, be arranged to occupy an area of approximately the right shape to fill the available space (19 × 12 cm), and they must be neatly pasted to a single piece of white cardboard. The photomicrographs in a single plate must be labelled A, B, C, &c. Room should be left to include the legend below the photomicrographs on the plate rather than in a separate list of plates.

[*continued on p. iii of cover*

Studies in Extension Growth

III. A RECORDING AUXANOMETER FOR PROLONGED OPERATION AT A RESOLUTION OF TEN MINUTES AND FIVE MICRONS

D. B. IDLE

Department of Botany, University of Birmingham

Received 17 August 1958

SUMMARY

An auxanometer is described that has been specially designed to study the timing of fluctuations in the extension rate of two separate tissues. It is capable of recording the extension rate on paper strip for long periods, while the tissues add up to 15 cm. to their initial length.

INTRODUCTION

THE auxanometer described previously (Idle, 1955) could record the extension rate of an organ; it was some disadvantage that only 0·5 cm. of growth could be followed without resetting the machine, since the effects on the plant of that operation lasted for a considerable proportion of the total time span available, which in practice was about 10 to 15 hours.

This paper describes an apparatus that has been designed for the study of correlated phenomena in the extension rates of two tissues. The resolution is 10 minutes and 5 μ, over a total distance of 15 cm. Two identical auxanometers are synchronized from a common clock, so that the time relation between events in both tissues may be accurately determined.

The apparatus has been used to study the behaviour of etiolated hypocotyls of *Helianthus annuus*. A specimen result is given to illustrate the abilities of the machine.

THE AUXANOMETER

The operation of this machine is best understood by considering first how the part on the left of the diagram in Fig. 1 follows the growth of the plant, and then how it is that the recording part on the right successfully amplifies the movements of the former.

The machine is really a contact auxanometer in which the switch S is moved upwards as long as it is closed by upward pressure from the plant under observation. The motor winds the switch up on steel wire over pulleys. The wire is held in tension by springs which are not shown in the diagram, but which are inserted in the wire strands as they pass down behind the switch. The pulleys are 1 in. diameter and have two shallow grooves cut to take the wires, which are about $\frac{1}{2}$ in. apart. The switch S operates the relay, R_1,

through a transistor amplifier. This relay controls the alternating current supply to the motor, *M*. As soon as the motor has pulled the switch up far enough to open it, the motor stops. The apparatus then waits until the plant grows enough to close *S* again.

The problem of recording the movements of the auxanometer is really one of reproducing the motion of the switch in an amplified form. In this machine, a paper strip is made to move over a drum several hundred times faster than

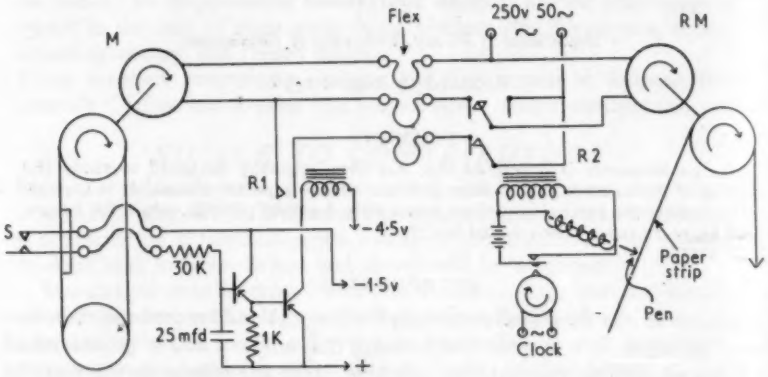


FIG. 1. The auxanometer. On the left is the apparatus that is attached to the plant, and on the right is the recording and timing mechanism. The two are joined by a flexible cable.

Description of Components

M and *RM*. Electric clock motors, 1 rev. per 24 hours, and 1 rev. per 4 minutes, respectively. Messrs. Chamberlain & Hookham Ltd., Birmingham.

R_1 and R_2. Post Office type 3000, each 500 ohms.

Transistors type OC71, Messrs. Mullard Ltd.

Clock. One revolution per 10 minutes.

The pen is a brass stylus, and the paper strip has a coating sensitive to brass, so that a black line is drawn continuously on the paper. The time mark is a displacement of this line. The paper strip is by Messrs. Recorder Charts Ltd., London.

The switch, *S*, is very similar to that described by Idle (1955) but clips on to the plant with rubber-lined jaws.

The capacitor in the input circuit of the transistor relay prevents chatter in R_1 when the switch is about to open or close.

the switch actually moves. As the paper passes, it is marked every 10 minutes so that when the strip is examined at the end of the experiment, the length of paper between each mark represents a magnification of the actual movement of the switch in the same time period. In the diagram, the paper is driven by the motor, *RM*, and is marked by a pen moving periodically on an impulse from the clock. Ignoring for a moment the relay, R_2, the recording motor is normally connected in parallel with the auxanometer motor, and, therefore, they both operate at the same time.

Here is a critical point: these motors maintain a complete synchronization, while at the same time providing magnification by virtue of their final shaft

speeds. The two motors start and stop together, and drive gear chains of different ratios to give the desired magnification.[1]

The relay R_2 operates at the same time as the pen, and ensures that the paper strip is moved on a little each 10 minutes, so that when the plant is not growing, elapsed time can be recorded.

The valve relay described earlier (Idle, 1955) would do as well as the transistor circuit, but the simplicity and trouble-free operation of the latter is an advance in technique of some importance. It makes the long experiments possible.

The twin auxanometers have been in operation for a year. There has been no breakdown and no maintenance. Batteries for the transistor relays last for about 2 months. The record for a period of 24 hours takes about 1 hour to plot graphically as rate with time, or longer if special techniques are used. The great advantage of this form of record is the great ease with which statistical operations such as totals for long periods and running totals may be accomplished by the use of ruler and dividers.

Since it is preferable to design an auxanometer for the particular problem in mind, this description has not included much detail of construction. The average extension rate of the tissue to be investigated determines to a large extent the magnification required, and the actual circumstances of observation of the particular plant must be known in advance so that the switch and its pulleys may have the optimum form.

SPECIMEN RESULT

The two auxanometers, working from a common timing clock, have been used to investigate the growth of the hypocotyl of *Helianthus annuus* seedlings.

The seeds were planted in moist sand in small jars, and left to germinate in darkness at about 20° C. in a compartment of an experimental darkroom. When the plants were about 4 cm. high, a jar was brought out into the darkroom and a plant attached to the apparatus. Manipulations were carried out under a dark-green photographic safelight. The darkroom was controlled at 22° C. by a double thermistor bridge. The thermal capacity of the room and controlling apparatus was such that the system cycled with a 3-minute period over a range of less than 0·5° C., and with a drift during 12 hours of less than 0·5° C. These measurements were made by thermocouples, and refer to the actual stem temperature of experimental *Helianthus* plants.

The two auxanometers may be attached to the same plant, but since the switches cannot share the same place on the hypocotyl, one has to go above the other. This means that the small length of stem between the effective

[1] They are electric clock motors by Messrs. Chamberlain & Hookham Ltd., of Birmingham. These motors have very short starting and stopping times, and the rotor speed and direction of rotation are determined. All other similar motors examined so far have a rotor too heavy, and a torque too low, to give the quick stop and start characteristic. The actual construction of the internal device for making the motors unidirectional is such that the operation of the auxanometer assumes that false starts are random in both motors, but this is a technical point that need not be elaborated. The performance of these motors has been very carefully examined.

points of contact of the switches is part of the observed growing zone of only one of the auxanometers. If the switches are clipped on near the plumular hook, this small zone has for some time a very small extension rate, and the two machines give indistinguishable records. After a time, however, the lower auxanometer is observing what is in effect only the lower part of the entire growing zone, while the upper machine observes all of it. The records are then not identical, since the extension rate of the entire zone is greater than that of a part. Graphs representing the extension rate of the entire zone, and the

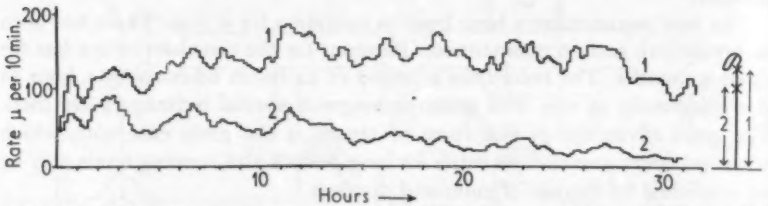


FIG. 2. Fluctuations of the extension rate of overlapping parts of the growing zone of an etiolated hypocotyl of *Helianthus annuus*.

rates of two parts of it, may be plotted from the records. It is also possible to arrange the two machines in cascade, so that each measures a part of the growing zone, and the rate of extension of the whole can then be found by addition.

Fig. 2 shows two records from overlapping growth zones of a *Helianthus* hypocotyl. The lower record is of the extension rate of the lower part of the hypocotyl, and gradually decreases to near zero as the cells come to the end of their grand period of extension. The upper record contains the events of the lower, plus those of the most active part of the whole stem. But the growth of the upper zone alone could be determined by difference.

It will be seen that in the early part of the graph the two records are nearly parallel, but later the rate of the lower becomes less perturbed, and although both results show a similar sequence of events, these are not always perfectly synchronous in the two records. Nor is the phase difference constant. This could mean simply that random fluctuations of the extension rate can be local, but closer study of many results suggests that the fluctuations are not random but periodic, and if this should be the case, then results such as these concern internal metabolic control and correlation phenomena in tissues. In this way studies of extension rates could give experimental evidence for the existence in tissues of metabolic inhomogeneities of the type described in the theoretical studies of Turing (1952), which lead to differential activity in space and time.

LITERATURE CITED

IDLE, D. B. (1955). Studies in extension growth. I. A new contact auxanometer. *J. Exp. Bot.* 7, 347–61.

TURING, A. M. (1952). The chemical basis of morphogenesis. *Phil. Trans. Roy. Soc.* B, 237, 37–72.

Comparative Enzyme Differentiation in Grass Roots

II. PEROXIDASE[1]

CHARLOTTE J. AVERS AND ROBERT B. GRIMM

Botany Dept., University of Miami, Coral Gables 46, Florida, U.S.A.

Received 12 November 1958

SUMMARY

Results of histochemical tests for peroxidase activity in seven species of grasses have been reported. The root epidermis of the festucoid grasses is characterized by rows of alternating shorter hair and longer hairless cells which can be recognized throughout their development. Peroxidase activity occurred in all the growing cells, but intensified reactions were observed in the hair cell initials in the basal portion of the elongation zone. The panicoid species have a root epidermis in which any cell seems capable of producing a root hair, and in these species all cells in the growing regions showed equal peroxidase activity. The close correlation between the differentiations of enzymes and cell types implies that physiological changes occur long before the morphological maturation of the tissue.

INTRODUCTION

PREVIOUS histochemical studies in grasses revealed a correlation between morphogenetic pattern and enzyme differentiation in the developing root epidermis (Avers, 1958; Avers and Grimm, 1959). These studies have been extended to include a number of enzyme systems for which adequate histochemical techniques were available. The present report concerns the relationship between peroxidase activity and epidermis differentiation in four festucoid and three panicoid species of grasses. As with acid phosphatase (Avers and Grimm, 1959) there was a definite correlation between enzyme differentiation and the developmental pattern of the root epidermis.

MATERIALS AND METHODS

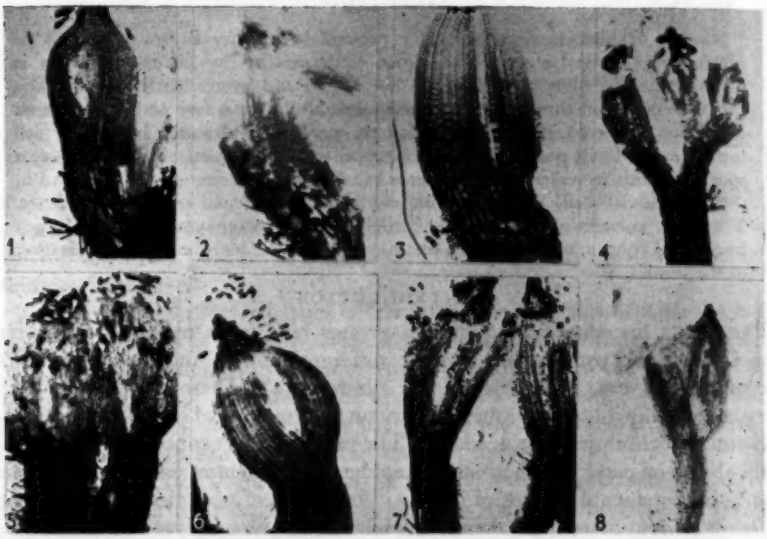
Seeds of *Poa trivialis*, *Phleum pratense*, *Festuca arundinacea*, *F. rubra*, *Panicum virgatum*, *P. antidotale*, and *Chloris gayana* were obtained from the U.S. Department of Agriculture and were germinated under the standard conditions previously described (Avers, 1958). Seedlings with primary roots 10–15 mm. long were selected and used unfixed in the histochemical tests. Gomori's technique (1952) was modified as follows: whole seedlings were submerged for 15–30 sec. in Solution I (0·5 per cent. benzidene, 0·2 per cent. sodium nitroprusside), and then for 40–60 sec. in Solution II (equal parts Solution I and 0·005 per cent. hydrogen peroxide). The roots then were

[1] This study was aided by a grant (G-4823) to the senior author from the National Science Foundation.

rinsed in distilled water, squashed and examined for blue and red-brown haemochromagens of peroxidase. It was found most desirable to use cold solutions although adequate results were obtained if they were kept at room temperature. The background colour of enzymatically inactive cells was light yellow. Control solutions consisted of the complete incubation media to which 0·05 per cent. potassium cyanide had been added just prior to treatment.

RESULTS

The festucoid grasses represented by *Festuca arundinacea*, *F. rubra*, *Poa trivialis*, and *Phleum pratense*, were characterized by root epidermis composed of longitudinal rows of alternating long and short cells. The long cells remain



FIGS. 1–7. Histochemical demonstrations of peroxidase activity in the root epidermis of seven species of grasses. Note the intensity of enzyme activity in the root-cap cells and in root hairs. The festucoid species: Fig. 1. *Phleum pratense*; Fig. 2. *Festuca arundinacea*; Fig. 3. *Festuca rubra*; Fig. 4. *Poa trivialis*. The panicoid species: Fig. 5. *Panicum antidotale*; Fig. 6. *Panicum virgatum*; Fig. 7. *Chloris gayana*.

FIG. 8. Example of the inactivation of peroxidase by 0·05 per cent.-KCN in control roots. The species shown is *Chloris gayana*.

All magnifications approximately ×50.

hairless at maturity while the shorter trichoblasts develop a root hair immediately prior to the cessation of cell elongation. These cell types were clearly recognizable during the major portion of their development, and probably began to differentiate in the basal portion of the meristem (Sinnott and Bloch, 1939; Goodwin and Avers, 1956). In the *Festuca* species there was a tendency for a trichoblast to alternate with two hairless initials, but in *Poa* and *Phleum* the majority of cell pairs consisted of one trichoblast and one

hairless initial. The results of the tests showed that peroxidase was active in all the growing cells of the festucoid epidermis, but greatly intensified activity occurred in the older trichoblasts (Figs. 1–4). These distributions were similar to the results with acid phosphatase for the *Festuca* and *Poa* species since the cells in this same physiological zone showed the differential enzyme activity (Avers and Grimm, 1959).

In *Phleum* the alternating trichoblasts which showed intensified peroxidase activity occurred in the basal portion of the elongating epidermis, whereas differential phosphatase activity had occurred in the more apically located enlarging cells (Avers, 1958). In *Phleum* the meristem occupies the first 300 μ of the root apex (exclusive of the root cap), and the zone of active cell elongation occurs 300–1,000 μ from the meristem apex (Goodwin and Avers, 1956). Differential acid phosphatase activity occupied the 300–500 μ zone, but peroxidase differentiation occurred 800–1,000 μ from the root apex. The zone of peroxidase differentiation was that in which the rate of cell elongation decreases from a maximum and begins to approach zero. Neither enzyme system showed differential activity in the zone of peak elongation rate, which is approximately 600–700 μ from the meristem apex (Goodwin and Avers, 1956).

The panicoid species, *Panicum virgatum*, *P. antidotale*, and *Chloris gayana* showed all growing cells of the epidermis to be equally enzyme-active (Figs. 5–7). This was similar to the acid phosphatase system where there was no inactivity in growing epidermal cells. With these species there is no particular pattern of hair and hairless cells, and any cell seems capable of producing a root hair (Row and Reeder, 1957). The apparently equal potentialities of all the growing epidermal cells appears to be correlated with the similarity in pattern (or lack of one) of enzyme differentiation.

No peroxidase activity was recorded for control roots (Fig. 8).

DISCUSSION

These results further substantiate the hypothesis that the morphogenesis of the root epidermis is characterized by the differentiation of enzymes at particular stages of development. The panicoid grasses showed no particular pattern of hair and hairless cells and no particular pattern of enzyme activity. In the festucoid species the trichoblasts and youngest root hair cells were enzymatically active while the alternating hairless initials showed less peroxidase activity in these same physiological areas. This is an indication of an early physiological change which is manifested later by morphological divergence of cell types within a single tissue.

In *Phleum pratense* the zones of differential acid phosphatase and peroxidase activities occurred in physiologically distinct areas. Indeed, they were separated by a narrow zone of cells elongating at the maximum rate. In this species acid phosphatase appears to be associated with an earlier phase of physiological differentiation than does peroxidase. However, in the two *Festuca* species and in *Poa* the reduction of peroxidase activity occurred in the same zone in which there had been inhibition of acid phosphatase. This

may be an indication of inherent physiological variation that occurs in species which show similar morphogenetic patterns. Further evidence of physiological divergence in morphogenetically similar tissue systems will be presented elsewhere.

LITERATURE CITED

AVERS, C. J. (1958). Histochemical studies of the differentiating root epidermis of *Phleum pratense*. *Amer. J. Bot.* **45**, 609.

—— and GRIMM, R. B. (1959). Comparative enzyme differentiation in grass roots. I. Acid phosphatase. Ibid. **46**, in press.

GOMORI, G. (1952). *Microscopic Histochemistry*. Univ. Chicago Press, Chicago, Illinois.

GOODWIN, R. H., and AVERS, C. J. (1956). Studies on roots. III. An analysis of root growth in *Phleum pratense* using photomicrographic records. *Amer. J. Bot.* **43**, 479.

ROW, H. C., and REEDER, J. R. (1957). Root-hair development as evidence of relationships among genera of Graminae. Ibid. **44**, 596. .

SINNOTT, E. W., and BLOCH, R. (1939). Cell polarity and the differentiation of root hairs. *Proc. National Acad. Sci. (U.S.)*, **25**, 248.

An Effect of pH and Bicarbonate on Salt Accumulation by Disks of Storage Tissue

R. G. HURD[1]

Department of Botany, King's College, London

Received 18 November 1958

SUMMARY

1. The uptake of potassium chloride by disks of several storage tissues has been determined from solutions at alkaline pH values and compared with that from solutions at pH 6·5. The stimulation of cation uptake under the alkaline conditions which has previously been obtained with beet tissues was observed with some of these tissues to a greater or lesser extent.

2. It is concluded that it is the accumulation of HCO_3^- anions present in alkaline solutions which generally promotes the uptake of cations; tissues which do not show the 'pH effect' accumulate very little HCO_3^- in the presence of the Cl^- anion.

3. In some cases the alkaline pH values also stimulated anion uptake. This was especially true in artichoke in which the absorption of both ions was stimulated by approximately the same amount. It is concluded that this represents either a true pH effect or a direct effect of HCO_3^- anions on the overall salt accumulation mechanism rather than an indirect effect through organic acid synthesis.

4. The experiments reveal a cation/anion ratio of approximately 1:1 when the uptake of HCO_3^- ions is taken into consideration.

It is suggested that this ratio may be more frequent than is generally accepted and that the uptake of CO_2 (converted to an anion in the tissue) may take the place of HCO_3^- ions at lower pH values.

5. The deleterious effects of HCO_3^- frequently observed on growth contrast with the stimulations of salt absorption obtained here; the rapid increase in the concentration of cations and/or organic acid anions associated with HCO_3^- uptake may in some way be responsible for this.

INTRODUCTION

In an earlier investigation (Hurd, 1958) the influence of pH on the uptake of KCl by beet storage-tissue disks was studied, and it was found that the large stimulation of cation uptake at alkaline pH values (the 'pH effect') was associated with the absorption of bicarbonate ions. It was obviously important to investigate other tissues to see if perhaps beet was unique among storage tissues in this respect. Other investigators using different materials have not obtained any comparable 'pH effect', despite recent evidence that appreciable amounts of HCO_3^- ions can be accumulated by a number of plants (Vickery and Palmer, 1957) and the fact that HCO_3^- ions are normally found in alkaline solutions. Recent workers on the effect of pH on salt uptake (Jacobson *et al*,

[1] Present address: W.A.C.R.I., Tafo, Ghana.

1957) have been careful to avoid the presence of bicarbonate during their investigations, but in the majority of the earlier work it was ignored.

The presence of HCO_3^- ions in the medium has been known for some time to be deleterious to plants (Harley and Lindner, 1945). Recently this has been attributed to inhibition of cytochrome oxidase (Miller and Evans, 1956) which Robertson *et al* (1947) have shown to be an important component of the terminal oxidase system of beet. One might suppose that this inhibition of metabolism would result in a depression of uptake in the presence of bicarbonate ions instead of the stimulation obtained. The present paper describes a more detailed investigation of the 'pH effect' using several tissues. It is attempted to reconcile the stimulatory effects of bicarbonate on cation uptake which were obtained in most tissues with the more generally observed inhibitory effects on metabolism.

METHODS AND MATERIALS

All of the storage tissues used were purchased locally and were of unknown variety with the exception of the beetroot, in which the variety Crimson Globe was used.

The methods of tissue preparation, the apparatus involved, and the methods of analysis have already been described (Hurd, 1958). The KCl solutions were buffered with tris-hydroxymethylaminomethane (subsequently referred to as 'tris') at a concentration of 0·01 M. associated with sulphuric acid; except in one of the experiments involving potato tissue, where 0·001 M. tris was used. The latter concentration was fairly efficient in maintaining the pH of the solution at its initial value although at high pH values the continual air stream through the solutions resulted in the retention of the CO_2 as HCO_3^- and the pH was lowered by up to one unit over the experimental period. Much smaller reductions in the pH value were obtained using 0·01 M. tris in solutions at an initial pH value of 8·5.

The uptake of HCO_3^- ions has been estimated by calculation from the decrease in HCO_3^- ion concentration of the solution. Bicarbonate may have been removed from the solution either by uptake into the tissue or by combination with H^+ ions released from the cells in exchange for the external cation, which causes CO_2 to be produced and lost to the atmosphere. However, it has been shown that the second alternative does not take place appreciably, at least in beet (Hurd, 1958). $^{14}C^-$ labelled bicarbonate was supplied and it was demonstrated that the loss of HCO_3^- from the solution (determined manometrically) was equivalent to that accumulated, as calculated from the radioactivity in the tissue after making an allowance for the drop in external activity due to exchange with respiratory CO_2.

RESULTS

'pH effect' in different storage tissues. The uptake of KCl by washed potato disks was determined at pH 8·5 and pH 6·5, the two pH values which were mainly used to demonstrate the 'pH effect' in beet. It was found in a

preliminary experiment that with salt at a concentration of 0·0024 M. (that used successfully for beet) and 0·00024 M., no effect of pH was obtained after 6 hrs. and 24 hrs. uptake (Table I). Uptake from solutions containing salt at a concentration of 0·024 M. proved difficult to estimate accurately as the change in external concentration was very small. However, there was no indication of a stimulation at the higher pH value from this concentration of salt. There was some inhibition of salt uptake at both the lower external concentrations due to the presence of the buffer, as shown by a direct comparison

TABLE I

The uptake of salt (μ eq./g. fresh wt.) by potato disks from solutions of potassium chloride at pH 6·5 and 8·5, at 25° C.

Uptake solution	Time in hours	pH 8·5		pH 6·5		No buffer	
		K^+	Cl^-	K^+	Cl^-	K^+	Cl^-
0·0024 M. KCl+0·01 M. tris and H_2SO_4	6	12·0	10·0	7·0	9·0	10·0	10·0
	24	22·5	28·5	10·0	23·0	24·5	30·0
0·00024 M. KCl+0·001 M. tris and H_2SO_4	6	4·5	5·5	4·0	5·5	6·0	7·5
	24	15·0	17·5	11·0	17·5	15·0	19·5

with unbuffered solutions at pH 6·5 (Table I). It is unlikely that a similar inhibition is masking a 'pH effect' at pH 8·5 since the figures obtained for the control at pH 6·5 and the buffered solution at pH 8·5 were closely alike in this and other similar experiments. Unless the inhibition and stimulation at the alkaline pH exactly counterbalance, which is unlikely, this would not occur. It is presumably the ionic form of tris which inhibits uptake since at pH 6·5, where the effect of the buffer is apparently greatest, 95 per cent. is dissociated, whereas at pH 8·5 only about 30 per cent. is dissociated.

In view of the absence of a 'pH effect' on potato, other storage tissues were examined in the form of disks and given the same pretreatment as potato and beet. These included white (sugar) beet, artichoke, parsnip (phloem), carrot (xylem and phloem), and swede (outer xylem). Xylem tissue of carrot behaved very similarly to the phloem and the results obtained with it are not recorded here. A simple comparison was made first between red beet and sugar beet (Table II).

The other tissues were compared with another sample of red beet on a subsequent occasion, which accounts for the quantitative difference in the two sets of results for red beet, although the disks on the two occasions behaved in qualitatively the same way. From Tables I and II it appears that the tissues differ considerably in the response of salt uptake to change in pH. Parsnip showed the greatest effect of pH on cation uptake, followed by red and white beet, carrot xylem and phloem, and artichoke, in descending order of magnitude; uptake by swede was unaffected by the different pH values, although, as in potato, tris buffer seemed to inhibit absorption of salt at the acid pH.

TABLE II

The uptake of salt (μ eq./g. fresh wt.) by red and white beet from solutions of potassium chloride at pH 6·5 and 8·5 at 25° C.

Tissue	Time in hours	pH 8·5		pH 6·5		No buffer	
		K^+	Cl^-	K^+	Cl^-	K^+	Cl^-
Red beet.	6	35·0	13·0	21·5	14·5	21·5	14·0
	24	101·5	53·0	73·0	53·0	60·0	46·5
Sugar beet	6	36·5	7·5	23·0	8·5	24·0	8·0
	24	106·0	43·5	52·5	28·5	55·5	29·0
Red beet.	6	25·5	6·5	12·0	5·0	9·0	4·0
	24	74	26·5	35·0	18·5	30·5	20·0
Artichoke	6	18·5	10·0	8·5	11·5	11·5	12·5
	24	50·0	47·5	24·5	40·5	28·5	34·5
Parsnip	6	36·5	2·5	6·0	4·5	9·5	5·5
	24	84·0	17·0	29·5	25·5	28·0	19·5
Carrot (phloem)	6	14·0	4·5	6·0	4·5	6·5	6·0
	24	46·0	17·5	18·0	18·0	19·0	15·5
Swede (outer uniform region)	6	23·0	17·5	14·0	11·5	18·0	13·5
	24	53·5	51·0	34·0	38·5	54·0	46·0

It was noted in the earlier investigation (Hurd, 1958) that the higher pH had a small but definite stimulatory effect on anion absorption by red beet. This has also been noticed in some of the other tissues used here, particularly artichoke where stimulation of anion uptake was observed after an experimental period of 24 hours, resulting in an approximately 1:1 ratio of cation to anion uptake at pH 8·5.

An insufficient number of tissues was examined to indicate any definite connexion between morphological nature and the responses to pH, although it seems from the results obtained that root tissues are more responsive than stem tissues. It also appears that those tissues not exhibiting a 'pH effect' are more sensitive to the presence of tris ions, as can be seen by comparing the uptake of cations at pH 6·5 in the presence and absence of the buffer (Tables I and II).

Uptake from $KHCO_3$ solutions. It has previously been shown that the 'pH effect' in beet is due to the presence of bicarbonate ions in the alkaline solutions. In accordance with this conclusion the uptakes of K^+ from KCl at pH 8·5 and from $KHCO_3$ at about the same pH were found to be approximately the same. Even when the pH value of the $KHCO_3$ was reduced to pH 6·2 with H_2SO_4 a stimulation was still observed, presumably due to the uptake of HCO_3^- remaining in solution. (Theoretically, hardly any HCO_3^- ions would remain, but in fact the equivalent of about 0·0007 M. was found in a control solution after aeration with ordinary air for the experimental period.)

The uptake from KCl and $KHCO_3$ solutions was measured in some of the other tissues for comparison with beet. It was thought probable that those not demonstrating the 'pH effect' would exhibit no stimulation of K^+ uptake from $KHCO_3$ relative to KCl at pH 6·5. This proved to be the case, and Table III gives the results of an experiment in which the uptakes of salt by red beet

TABLE III

The uptake of ions (μ *eq./g. fresh wt.*) *from 0·0024 M. KCl and 0·0024 M.* $KHCO_3$ *by various tissues, at 25° C.*

Time in hours	Parsnip				Beet			
	KCl		$KHCO_3$		KCl		$KHCO_3$	
	K^+	Cl^-	K^+	HCO_3^-	K^+	Cl^-	K^+	HCO_3^-
24	21·5	13	73·5	76	20·5	13·5	34	29
48	42·5	30	134	131·5	28	17·5	57	48·5
72	58	41	181	176	30	20·5	66·5	56·5
96	68·5	48·5	220	216	30	21·0	71·5	65·5
	Swede				Potato			
24	52·5	50	55	52·5	7·5	10	7·5	11
48	86·5	83·5	88	86·5	18	23	18	17·5
72	118·5	112·5	108·5	109·5	23·5	30·5	22	22
96	141	130·5	122·5	127	No further uptake			

and parsnip were compared with those by swede and potato. K^+ uptake was greater from the $KHCO_3$ solution with beet and parsnip but not with potato or swede. Further, the relative stimulations in the former cases were of the same order as those obtained in alkaline KCl solutions. Fig. 1 is a histogram compiled from the figures presented which demonstrates this similarity in K^+ uptake from solutions of $KHCO_3$ and KCl at pH 8·5. Table IV indicates that the final cation level in beet and parsnip would probably have differed between the two salts in the same way as was found in the earlier investigation when comparing the final salt content of beet tissue after uptake from KCl at two pH values. This close similarity between uptake of K^+ from $KHCO_3$ and KCl at pH 8·5 is valuable confirmatory evidence that HCO_3^- ions are responsible for the 'pH effect'.

There was found to be a close relationship between the HCO_3^- lost from the solution and the K^+ taken up in all four of the above tissues. This is inevitable if the external solution is to remain in electrical equilibrium, unless there is an exchange for other ions from the tissue. Only the possibility that K^+ is taken up partly by exchange for H^+ is considered here. Sutcliffe (1954) showed that exchange of K^+ for Na^+ is slow in beet disks. The loss of HCO_3^- from the solution is believed to represent an accumulation in the tissue because of evidence previously mentioned involving the use of radioactive carbon.

It was also observed that during the absorption of salt by beet disks from $KHCO_3$ solutions the pH value of the external solution increased (Table IV).

This was presumably due to the gradual equilibration between HCO_3^- and the CO_2 of the air circulating through the solution. Under the conditions used, some of the HCO_3^- would be lost and replaced by OH^- until the pH was raised to such a level that the remaining HCO_3^- concentration was in equilibrium with the existing partial pressure of CO_2. Now, in contrast to this, if H^+ was

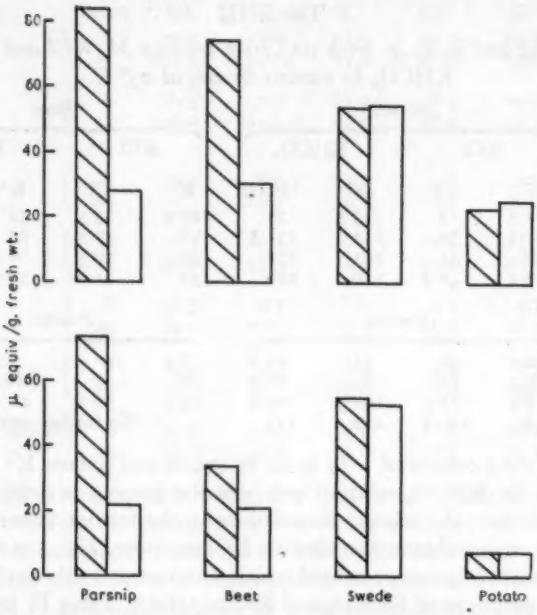


FIG. 1. Comparison of uptake during a 24-hour period of potassium from bicarbonate and chloride. Upper half: shaded blocks, K^+ uptake from KCl at pH 8·5; unshaded blocks, K^+ uptake from KCl at pH 6·2. Lower half: shaded blocks, K^+ uptake from $KHCO_3$ at pH 8·5; unshaded blocks, K^+ uptake from KCl at pH 6·5.

exchanged for K^+, the HCO_3^- would be effectively titrated, lowering the pH value. In the author's experience, the equilibration of solutions of HCO_3^- with CO_2 of the atmosphere is relatively slow, and it seems likely that if H^+ exchange occurred to the extent required to account for K^+ absorption from a potassium bicarbonate solution, the pH value of the medium would tend to decrease instead of increasing as was experimentally observed.

The approximate equivalence of uptake of anions and cations has been noticed in all the experiments described in this paper, when the uptake of HCO_3^- is taken into consideration (Tables III–VI).

Uptake from mixed $KHCO_3$ *and* KCl *solutions.* The final series of experiments involved estimation of the uptake of ions from mixed $KHCO_3$ and KCl solutions, using red beet tissue only, in an attempt to further elucidate the 'pH effect'. These solutions approach most closely the conditions found

TABLE IV

The uptake of ions (μ eq./g. fresh wt.) by red beet disks from solutions of KCl *and* $KHCO_3$ *in 24 hrs. at 25° C., and the initial and final pH values of the solutions*

Salt	Molarity	Salt uptake		pH values	
		K^+	Cl^- or HCO_3^-	Initial	Final
KCl	0·0012	19·0	7·5	6·1	5·3
	0·0024	30·0	22·0	6·2	5·2
$KHCO_3$	0·0012	33·0	37·5	7·3	7·8
	0·0024	41·5	39·5	7·5	8·0

TABLE V

The uptake of potassium, chloride, and bicarbonate ions (μ eq. g./fresh wt.) by beet disks from solutions containing various proportions of KCl *and* $KHCO_3$, *at 25° C.*

Treatment		5	4	3	2	1
Solution	KCl	0·0024 M.	0·0018 M.	0·0012 M.	0·0006 M.	..
	$KHCO_3$	..	0·0006 M.	0·0012 M.	0·0018 M.	0·0024 M.
Initial pH value		6·1	6·9	7·3	7·8	pH 7·5

Treatment	K^+ uptake		Cl^- uptake		HCO_3^- uptake		Total anion uptake	
	24 hrs.	55 hrs.	24 hrs.	55 hrs.	24 hrs.	55 hrs.	24 hrs.	55 hrs.
1	31·0	51·5	..	..	26·5	44·0	26·5	44·0
2	35·0	68·5	15·0	32·5	15·0	23·5	30·0	56·0
3	34·5	60·5	15·0	29·5	16·0	24·0	31·0	53·5
4	33·0	58·0	12·5	26·5	16·0	23·5	28·5	50·0
5	25·0	43·5	11·0	26·0	..	..	11·0	26·0

TABLE VI

The uptake of ions (μ eq./g. fresh wt.) by beet disks from 0·024 M. KCl. *and 0·024 M.* $KHCO_3$

Time of uptake (hrs.)	0·024 M. KCl		0·024 M. $KHCO_3$	
	K^+	Cl^-	K^+	HCO_3^-
7	56·5±2	..	53·0±2	43·0±8
30	119·5±4	..	136·5±3	130·0±12
80	179·5±8	141·0*	256·5±5	223·0±16

* 2 identical replicates.

in 'KCl at pH 8·5' solutions, when the concentrations of KCl and $KHCO_3$ are suitably adjusted. It can be calculated that at equilibrium in air a solution at pH 8·5 contains approximately 0·0017 M. HCO_3^- (depending on the partial pressure of CO_2 in the air); therefore a mixture containing 0·0018 M. $KHCO_3$ and 0·0006 M. KCl would, apart from the reduced Cl^- concentration, resemble the alkaline buffered 0·0024 M. KCl. The uptakes from solutions containing this mixture and 2 other ratios of KCl/$KHCO_3$, all with a K^+ concentration of 0·0024 M., were compared with those from the single salts at the same K^+ concentration. The results are given in Table V and it is obvious that all of

the mixed salts are superior to $KHCO_3$ alone in promoting K^+ uptake. The presence of any of the three concentrations of HCO_3^- appeared to stimulate Cl^- uptake, whereas HCO_3^- uptake was reduced by an amount which was relatively independent of HCO_3^- concentration or the ratio of Cl^- to HCO_3^-. This concentration independence of HCO_3^- uptake from the mixed salt solution

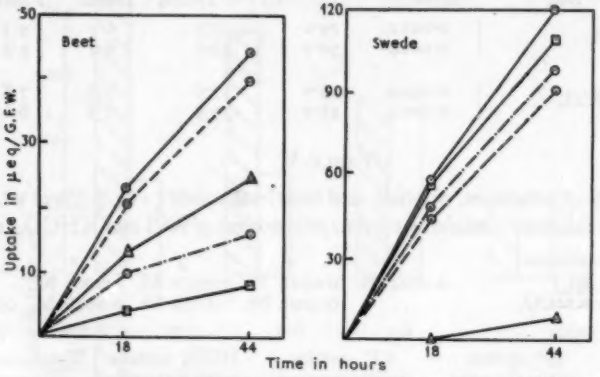


FIG. 2. Comparison of uptake from 0·0012 M. $KHCO_3$ + 0·0012 M. KCl solution with that from 0·0024 M. $KHCO_3$ alone and 0·0024 M. KCl alone at 25° C. —⊙— K^+, —△— HCO_3^- and —⊡— Cl^- uptake from $KHCO_3$ + KCl, ——⊙—— K^+ uptake from $KHCO_3$, —·—⊙—·— uptake from KCl.

suggests that HCO_3^- can be accumulated from salt solutions at pH 6·5 in which its concentration is normally very low, and it may account for the excess cation uptake normally found at this pH value (Table II).

Uptake by beet and swede from a solution containing KCl and $KHCO_3$ in the ratio 1:1 was compared with that from the single salts; in all three solutions the total K^+ concentration was 0·0024 M. (Fig. 2). The most notable feature of this experiment was the marked preference shown by the swede to accumulate Cl^- rather than HCO_3^- from the mixed salt solution. Assuming that the loss of HCO_3^- from the solution can be interpreted as an uptake of that ion by the tissue, then this failure to accumulate HCO_3^- in the presence of Cl^- could explain the lack of a 'pH effect' in this tissue. In KCl solution very little of the available HCO_3^- at pH 8·5 is taken up and no additional K^+ can be accumulated. This explanation may apply to potato also but in an experiment designed to demonstrate this the uptake of salt by the material available was too low to provide accurate data.

Uptake from high salt concentrations. In the previous investigation (Hurd, 1958) it was noted that the uptake from a 0·024 M. KCl solution (10 times the concentration normally used) was not initially affected by the pH value of the solution, but stimulation was observed later. Using 0·024 M. $KHCO_3$ solutions the same effect was obtained (Table VI).

The saturation level of K^+. Although the saturation level of cations attain-

able by the disks is different at the 2 pH values, being much higher from a solution at pH 8·5 than at pH 6·5, it was not found possible to increase the K^+ concentration within disks once they had been filled in pH 6·5 solutions by placing them in solutions at pH 8·5. Disks kept in KCl and $KHCO_3$ solutions also had widely differing final cation levels after 80 hours (see Table VI), those from $KHCO_3$ solutions being much higher than from KCl solutions. Again, after filling disks from one solution, bathing them in the alternative solution neither increased nor decreased the final K concentration, no matter which solution was applied first. It appears, in general, that once disks are saturated with K^+ from one solution they cannot be induced to take up any further K^+.

DISCUSSION

Further consideration of the 'pH effect'. Work with red beet established that a part of cation uptake at alkaline pH values is associated with organic acids produced from absorbed HCO_3^-. In the experiments described above, there was, in many cases, a close resemblance between the results obtained using alkaline KCl solutions and those using $KHCO_3$ solutions, indicating that it is the presence of HCO_3^- in the alkaline solutions which is associated with the 'pH effect' described in beet. The 'pH effect' shown by some of the other tissues examined is assumed to be related to the synthesis of organic anions from HCO_3^-, in the same way as has been shown for red beet.

The effect of pH on the absorption of salt by artichoke tissue differed from that observed with other materials in that both cation and anion absorption was stimulated by a comparable amount at alkaline pH (Table II). This fact suggests that the explanation of cation stimulation in artichoke may be different, at least in part, from that outlined above, since the excess cations absorbed are balanced by Cl^- rather that by organic anions. Although the data are inadequate to explain completely the situation in artichoke, they are consistent with the idea that either bicarbonate ions or pH *per se* stimulate salt uptake by directly influencing the cation or anion accumulatory mechanism, or both. It is interesting to note that none of the other tissues showing 'pH effects' show a significant stimulation of Cl^- absorption at alkaline pH (Table II). In these tissues, in contrast to artichoke, it seems that any influence of HCO_3^- or pH directly on cation uptake is less important than the indirect effect via organic acid synthesis.

In potato and swede, no stimulatory effects of HCO_3^- on cation uptake have been obtained. It is possible that, whereas in those tissues demonstrating the 'pH effect' anion uptake is limiting total salt absorption under the conditions of the experiment, in swede and potato it is cation uptake which is limiting. The presence of additional absorbable anions would not in this case be expected to result in further cation uptake. It should be possible to verify this using other salt solutions in which anion absorption is likely to be limiting, e.g. a solution of potassium sulphate. Alternatively, the total uptake of salt may be limited by the capacity of an anion accumulatory system, common

to chloride and bicarbonate. In either case, the almost complete suppression of HCO_3^- uptake in swede which was observed in the mixed solution is presumably the result of extreme competition with chloride, and this alone may be responsible for the absence of a 'pH effect' in this tissue. It seems rather significant that two tissues (potato and swede) which exhibit suppression of bicarbonate in presence of chloride are those which show no 'pH effect'. It should be possible, using other combinations of salts, to elucidate the situation further. The grouping of tissues into those demonstrating a 'pH effect' and those not doing so probably depends to some extent on the particular salt solutions employed, and using mixtures from which anions and cations are absorbed at different rates, different groupings may be obtained.

It is possible that in red beet the absence of a 'pH effect' when uptake occurs from solutions containing salts at a concentration of 0·024 M. is due to cation uptake being limiting, in contrast to limiting anion absorption occurring at the lower concentrations. The 'pH effect' observed after some time when uptake occurs from a 0·024 M. solution may then have a different explanation from the 'pH effect' at 0·0024 M. After a period of salt uptake the internal concentration of salt begins to influence the rate of uptake (Sutcliffe, 1952). Since the tissue in a $KHCO_3$ solution can attain a higher potassium concentration than it can in a KCl solution (Hurd, 1958), this influence is first observed in the KCl treatment, and appears as a delayed 'pH effect'.

This second aspect of the 'pH effect' involving the different potassium ion concentrations attainable in different potassium-containing salt solutions was not closely investigated, but the experiments in which beet disks were transferred from one solution to another provide some preliminary information. It is surprising that once disks have been filled from a KCl solution at pH 6·5, they will accumulate no further K^+ on transference to either KCl at pH 8·5 or to $KHCO_3$ solutions. This indicates that bicarbonate ions do not have a direct effect on the accumulatory mechanism in this tissue. It also indicates that the number of ions in a tissue in some way limits further uptake, possibly by an osmotic effect as mentioned previously (Hurd, 1958). The difference in the final potassium concentration after uptake from KCl and bicarbonate solutions could be explained by supposing that bicarbonate ions are incorporated into existing molecules, e.g. organic acids, with the result that only one ion is effectively accumulated from a $KHCO_3$ molecule, compared with two from KCl. This seems unlikely to be the whole explanation since there is poor correlation between the differences observed after uptake from the two salts and that predicted on the basis of the above argument. The total changes in osmotic pressure through the absorption of salt over the course of uptake only amount to 1–2 atmospheres. Since this change is spread over several days it is obvious that changes in other osmotically active components of the system, particularly the carbohydrate reserves, are likely to be more extensive and overshadow smaller effects due to changes in salt content.

An alternative explanation which is also compatible with the experimental findings is that the anion accumulatory mechanism (in the case of KCl up-

take) becomes blocked after a period of salt uptake preventing any further anion or cation absorption (Sutcliffe, 1954). The internal concentration at which the block occurs could quite possibly vary with different anions. Once the mechanism is blocked, transference to a different salt solution, from which anions are accumulated by the blocked mechanism, would not result in any further ion uptake regardless of the normal level attainable when a tissue is placed in the second solution from the start. It is likely that Cl^- and HCO_3^- ions are accumulated by the same or by closely related mechanisms because of the noticeable inhibition of HCO_3^- uptake in the presence of Cl^- with beet and other tissues. It would be interesting to see if transference to solutions of salts containing other anions than HCO_3^- after treatment in KCl solutions would result in any further metabolic uptake. This might provide information as to the independence or otherwise of different anion accumulatory systems, since an entirely distinct mechanism might be expected to be unaffected by previously accumulated ions. It is possible that in other situations the cation accumulating mechanism may be limiting uptake, opening up the possibility of testing the independence of different cation accumulating systems in a similar manner.

Equal cation and anion accumulation. It has been observed that there is an approximately equal metabolic uptake of cations and anions from both single and mixed salt solutions when allowance is made for the uptake of HCO_3^-. This equivalence is clearly demonstrated in cases where HCO_3^- is not accumulated as in swede and potato during uptake from mixed KCl and $KHCO_3$ solutions where an equal uptake of K^+ and Cl^- was observed.

Three factors may contribute to the fact that one seldom obtains an exact equality of uptake: (*a*) limitations in the accuracy of the estimation, (*b*) exchange of similarly charged ions, (*c*) uptake of CO_2 as opposed to HCO_3^-. The fact that the ratio nevertheless approximates to unity demonstrates the limited importance which may be attached to these complicating factors. Although the importance of HCO_3^- ions in securing equal cation/anion uptake is evident in the tissues investigated here it cannot be involved in some cases of apparently unequal uptake reported in the literature. Such observations have been made at pH values where little or no HCO_3^- ions can be present (Jacobson, 1955). There are also a few instances of excess anion uptake where the uptake of HCO_3^- ions would only accentuate the unbalance of ions accumulated (Ulrich, 1941).

It may be proposed that at low pH values CO_2 can be accumulated and converted to organic anions to compensate the excess cations taken up; these then enter in exchange for H^+ ions. Jacobson (1955) obtained limited evidence in agreement with this and Poel and Graham (1956) working at acid pH values also suggest that the uptake of CO_2 may play an important part in the cation/anion uptake equilibrium.

With respect to cases of excess anion uptake from single salt solutions it may be noted that the excess is never large and the number of salts from which it occurs is small, always involving a slowly accumulated cation (Ulrich, 1941).

It is possible that the small excess of anions involved exchange for organic (or other) anions within the cell. It has been shown by Steward, Stout, and Preston (1940) that HCO_3^- anions are exchanged for external anions during excess anion uptake. By ensuring that equal amounts of oppositely charged ions are accumulated, the plant cell automatically maintains electrical neutrality and a relatively constant pH value. The alternative proposal made by Ulrich (1941) starts by assuming that unequal uptake occurs and that the cell counteracts the inequality by producing or disposing of organic acids. The present scheme has the advantage that it does not require a mechanism for assessing and counteracting this upset in the equilibrium induced by unequal uptake. Burström (1945) has modified Ulrich's view by supposing that although unequal uptake may occur into the cytoplasm where adsorptive processes are involved, during the accumulatory process into the vacuole those cations not associated with anions are associated with synthesized malic acid so that equal uptake results. This combines the two views previously expressed and allows for the metabolism of ions, e.g. those containing nitrogen, before they are accumulated into the vacuoles. This was considered to be an explanation of the excess anion accumulation observed from solutions of nitrates. The only difference between Burström's and the present author's views is that Burström suggests that a 'mother substance' acts as a source of malic acid, whereas it is here proposed that external HCO_3^- ions can provide that source.

There are some other reports in which the uptake of HCO_3^- or CO_2 is believed to have a balancing effect on the ratio of cations to anions taken up. Scharrer and Jung (1957) have followed the uptake of salts in sand culture by sunflower and maize. They have observed that the cation/anion ratio within the plant is reasonably constant despite the uptake from different mixtures of salts at different concentrations. This indicates that a constant ratio of cations/anions must have been accumulated in all cases although not necessarily in a 1:1 ratio. They found that the presence of HCO_3^- modified this ratio and they concluded that HCO_3^- must be accumulated. They showed that ^{14}C-labelled bicarbonate was taken up by the plant although their technique did not allow them to confirm the amount of uptake and hence the maintenance of the cation/anion ratio. Scharrer and Jung also found an equivalent uptake of acetate and its associated cation from the simple salt solution. This interesting observation may perhaps be explained by assuming that the organic acid formation associated with HCO_3^- and CO_2 uptake is suppressed in the presence of large amounts of accumulating organic acid anions. This would preclude any additional cation uptake due to CO_2 or HCO_3^- uptake and would result in equal uptake of the two ions under observation.

The leaves of water plants also accumulate HCO_3^- ions in association with an equal amount of cations (Arens, 1936). Lowenhaupt (1956) reviewing this work suggested that equal uptake may also occur in land plants although the evidence then available suggested that CO_2 might be entirely responsible for making up the balance.

The deleterious effects of HCO_3^- *ions.* In none of the tissues investigated was the presence of HCO_3^- ions found to be deleterious to the tissue as far as could be determined from analyses of the salt accumulation over periods of several days. Considering the harmful effects frequently observed on growth this may seem surprising; however, one explanation is that the concentrations at which these harmful effects have been obtained are usually higher than those used here. Hassan and Overstreet (1952) noted that 0·01 M. $NaHCO_3$ was slightly toxic to the growth of the radicle of freshly germinated radish seeds whilst the 0·05 M. salt was decidedly toxic. Miller and Evans (1956) also noted inhibitory effects on cytochrome oxidase activity at a bicarbonate concentration of 0·05 M. in mitochondrial preparations and whole roots. However, they did not show that all concentrations of HCO_3^- ions are deleterious since they appear to have employed solutions containing HCO_3^- ions in some of their controls (they used tris-HCO_3 buffers). Neither do they record that their mitochondrial extraction procedure excluded HCO_3^- ions. Stolwijk and Thimann (1957) considered that it was the CO_2 rather than HCO_3^- which was responsible for the inhibitory effects on growth they obtained using peas and oats.

There is no doubt, however, that in spite of any stimulatory effects it might have on salt uptake, HCO_3^- is generally deleterious to plant growth, whether this is observed as a reduced growth-rate (Brown and Wadleigh, 1954) or as an effect on the metabolism of the whole plant, as in alkaline chlorosis (Harley and Lindner, 1945). Hassan and Overstreet (1952) have observed both the stimulation of accumulation-rate and the depression of growth-rate during uptake of $NaHCO_3$ by radish radicles, so the stimulation of salt uptake is not restricted to the non-dividing vacuolated cells of the red beet used here. It is possible that these two effects of HCO_3^- ions are related and that it is the increased content of the cations and/or the organic acid anions in the cells which is responsible for the deleterious effects observed on growth.

ACKNOWLEDGEMENTS

I wish to thank Dr. J. F. Sutcliffe for his supervision of this work and for reading the manuscript of this paper which is extracted from a Ph.D. thesis in the University of London. I also wish to thank Professor T. A. Bennet-Clark for his encouragement and the D.S.I.R. for a maintenance grant which made it possible for this research to be undertaken.

LITERATURE CITED

Arens, K. (1936). Physiologisch polarisierter Massenaustauch und Photosynthese bei submersen Wasserpflanzen II Die $Ca(HCO_3)_2$ Assimilation. *J. wiss. Bot.* **83**, 513–60.

Brown, J. W., and Wadleigh, C. H. (1954). Influence of sodium bicarbonate on the growth and chlorosis of garden beets. *Bot. Gaz.* **116**, 201–9.

Burström, H. (1945). Studies on the buffer systems of cells. *Arkiv. für Botanik*, **32A**, 1–18.

Harley, C. P., and Lindner, R. C. (1945). Observed responses of apple and pear trees to some irrigation waters of north central Washington. *Proc. Amer. Soc. Hort. Sci.* **46**, 35–44.

HASSAN, M. N., and OVERSTREET, R. (1952). Elongation of seedlings as a biological test of alkali soils. I. Effects of ions on elongation. *Soil Sci.* **73,** 315–26.

HURD, R. G. (1958). The effect of pH and bicarbonate ions on the uptake of salts by disks of red beet. *J. Exp. Bot.* **9,** 159–75.

JACOBSON, L. (1955). Carbon dioxide fixation and ion absorption in barley roots. *Plant Physiol.* **30,** 264–9.

——, OVERSTREET, R., CARLSON, R. M., and CHASTAIN, J. A. (1957). The effect of pH and temperature on the absorption of potassium and bromide by barley roots. *Plant Physiol.* **32,** 658–61.

LOWENHAUPT, B. (1956). The transport of calcium and other cations in submerged aquatic plants. *Biol. Rev.* **31,** 371–95.

MILLER, G. W., and EVANS, H. J. (1956). Inhibition of plant cytochrome oxidase by bicarbonate. *Nature,* **178,** 974–6.

POEL, L., and GRAHAM, J. S. D. (1956). Carbon dioxide fixation and mineral absorption in barley roots. *Revue des Ferm. et des Ind. Alim.* **11,** 200–2.

ROBERTSON, R. N., TURNER, J. S., and WILKINS, M. J. (1947). Studies in the metabolism of plant cells. V. Salt respiration and accumulation in red beet tissue. *Aust. J. Exp. Biol. Med. Sci.* **25,** 1–8.

SCHARRER, K. VON, and JUNG, J. (1957). Über den einfluß Verschiedener anionen auf die Aufnahme von calcium, natrium und kalium. *Plant and Soil,* **9,** 49–63.

STEWARD, F. C., STOUT, P. R., and PRESTON, C. (1940). The balance sheet of metabolites for potato disks showing the effect of salts and dissolved oxygen on metabolism at 23° C. *Plant Physiol.* **15,** 409–47.

STOLWIJK, J. A., and THIMANN, K. V. (1957). On the uptake of carbon dioxide and bicarbonate by roots, and its influence on growth. Ibid. **32,** 513–19.

SUTCLIFFE, J. F. (1952). The influence of internal ion concentration on potassium accumulation and salt respiration of red beet tissue. *J. Exp. Bot.* **3,** 59–76.

SUTCLIFFE, J. F. (1954). Cation absorption by non-growing cells. *Soc. Exptl. Biol. Symp.* **8,** 325–42.

ULRICH, A. (1941). Metabolism of non-volatile organic acids in excised barley roots as related to cation-anion balance during salt accumulation. *Amer. J. Bot.* **28,** 526–37.

VICKERY, H. B., and PALMER, J. K. (1957). The metabolism of the organic acids of tobacco leaves. XIII. Effect of culture of excised leaves in solutions of potassium bicarbonate. *J. Biol. Chem.* **227,** 69–81.

The Effect of Some Growth-regulating Substances on the Development of *Sclerotium rolfsii* (Sacc.)[1]

S. LAVEE[2]

Division of Horticulture, Agricultural Research Station, Rehovot, Israel

Received 12 December 1958

SUMMARY

Experiments were carried out in order to establish the effect of some growth regulators on the development of *Sclerotium rolfsii*. Triethanolamine was found to be physiologically active; coumarin, maleic hydrazide, and triethanolamine cause pronounced inhibition of germination of sclerotia and reduction in the number of sclerotia per culture. Coumarin in concentrations of 50–100 p.p.m. increased the size of the developing sclerotia. α-Naphthylacetic acid inhibited germination but had a promoting effect on the development of sclerotia. In concentrations of 50–100 p.p.m. it caused a more compact and dense development of the mycelium and lowered the rate of growth.

INTRODUCTION

THE effect of growth-substances on the development of micro-organisms has already been intensively studied (Crowdy, 1948; Davis and Dimond, 1953). It seemed therefore of interest to investigate the effect of growth-inhibiting substances on the development of *Sclerotium rolfsii* (Sacc.) which attacks apple-trees in addition to various vegetables in Israel in late summer (Peleg, 1945, 1947; Lavee, 1955). In this period the natural inhibitors in the trees are believed to increase in quantity (Samish, 1954).

S. rolfsii is the imperfect form of a Basidiomycete. It grows rapidly in culture and hence is an excellent subject for determining the effect of inhibitors on its development.

The size of the sclerotia on a culture is apt to vary according to growth conditions (Nakata, 1927; Cursi, 1932) and stands in direct relation to the number formed on the media.

METHODS

The experiments described in this article were conducted in standard petri-dishes (90 mm. diameter), containing 13·5 ml. media. The medium for all our cultures was a potato glucose agar (Rosen and Shaw, 1929), containing 200 g. potatoes, 20 g. glucose, 20 g. agar per l. of water. The potatoes were cooked until soft in half of the water and then squeezed through cheese cloth. To this we added the substances whose action on *S. rolfsii* was to be investigated. All the media were sterilized for 20 min. at a pressure of 15 lb./sq. inch.

Media containing coumarin were prepared after the independent effect of

[1] Publication of the Agricultural Research Station, Rehovot, 1957, Series No. 191-E.

[2] Present address: Dept. of Botany, Univ. of Wisconsin, Madison, Wisc., U.S.A.

triethanolamine on germination had been established by weighing the amount of inhibitor for each culture separately.

The maleic hydrazide (MH) used was the commercial product MH40, which is the Na-salt, and is readily soluble in water. The concentrations of MH recorded were always calculated for the pure hydrazide. α-Naphthylacetic acid (NAA) was dissolved in ethanol which was expected to evaporate during sterilization. All media were adjusted to an initial pH of 6·7. No buffer was used as the fungus greatly reduces pH during development (pH 2) whether buffer was added or not (Lavee, 1955).

The fungus was isolated from the roots of an infected apple-tree. The sclerotia were washed under running water and then rinsed with sterilized water. No further surface sterilization of the sclerotia was done. Immediately after washing the sclerotia were placed in the middle of plates containing the media. The developing mycelium covered the whole plate and new sclerotia were formed. Sclerotia for mother cultures were taken from clean uncontaminated parts of the plate. These cultures were grown in test-tubes on the same medium.

New mother cultures were prepared from the original isolate before each experiment. Generally sclerotia of one month were used as these were previously found to be most active in germination (Lavee, 1955).

For experiments with sclerotia of low viability in the sense of weak germination power and low growth-rate, sclerotia of 6–12 months were used.

The experimental cultures were started from a single sclerotium planted in the middle of the dish (so that one colony developed per dish), and grown at a constant temperature of 26–27° C. As the colony has a radial development one measurement was needed per plate at a time. Humidity was kept at a high and constant level by introducing open plates with water into the incubator. The cultures were kept in the dark except when being measured.

Results were obtained by measuring the diameter of the cultures at fixed intervals; time to reach various diameters were graphically determined; and by noting the first appearance (with binoculars) and final amount of sclerotia in the culture. All figures reported represent the average of at least ten cultures. At least three repetitions of each treatment were made at a time. The average was calculated from all ten plates undergoing the same treatment.

EXPERIMENTAL RESULTS

Series of media containing an increasing concentration of maleic hydrazide were prepared and inoculated. It could be seen that with increasing concentrations of the inhibitor, the cultures took a correspondingly longer time to complete their vegetative development and the number of sclerotia formed decreased (Table I).

From Table I it can be seen that inhibition of germination occurred but the rate of growth of the mycelium after full germination was not affected. In Table II it is shown that coumarin had a similar and stronger inhibitory effect on the initial development of the fungus.

With coumarin we note a critical concentration above which no development took place. This concentration was over 100 p.p.m. under our conditions (9 of the 10 sclerotia still germinated). The reduction in the number of developing sclerotia is even stronger with coumarin than with MH.

TABLE I

The influence of maleic hydrazide on the development of S. rolfsii

Concentration of maleic hydrazide in p.p.m.	Mean time in hours to reach diameter of 1 mm. (germination)	Rate of growth mm./hour after germination	Mean time in days to sclerotial initials	Mean number of sclerotia formed per plate
0	63	1·58	5	475
1	58	1·46	5	423
5	68	1·54	5½	413
10	71	1·62	6	420
25	71	1·66	6	367
50	74	1·54	6	333
100	82	1·46	7	290
L.S.D. at $P = 0{\cdot}05$	6	0·21	1	91

TABLE II

The influence of coumarin on the development of S. rolfsii

Concentration of coumarin in p.p.m.	Time in hours to reach diameter of 1 mm. (germination)	Rate of growth mm./hour after germination	Mean time in days to sclerotial initials	Mean number of sclerotia formed per plate
0	72	1·58	6	465
1	72	1·62	6	440
5	71	1·58	6	460
10	78	1·58	6½	457
25	86	1·50	7	347
50	113	1·54	12	220
100	161	1·54	16	165
500		no growth		
L.S.D. at $P = 0{\cdot}05$	10	0·03	2	48

The variability of the sclerotia in the experiment with the coumarin was somewhat lower. This explains the difference in growth and appearance of sclerotia in the control cultures of the two experiments represented in Tables I and II.

The sensitivity of *S. rolfsii* to MH is not constant, but is affected by the viability of the sclerotia.

In some of our experiments, where the development of the control cultures was slower than usual, the sensitivity to MH was much higher, as a consequence of lower viability of the mother sclerotia (Table III).

The critical concentration of coumarin varies too according to the viability of the sclerotia, as has been shown for MH. Very vigorous sclerotia succeeded

in germinating even on 500 p.p.m. coumarin, although these cultures did not reach normal development (Fig. 1).

TABLE III

The influence of maleic hydrazide on the development of S. rolfsii *of low viability*

Concentration of maleic hydrazide in p.p.m.	Mean time in days to reach diameter of 1 mm. (germination)	Rate of growth mm./hour after germination	Time in days to sclerotial initials
0	5	1·50	8
10	11	1·25	15
50	37	1·50	40
100	no growth		nil
L.S.D. at $P = 0·05$	2½	0·80	2½

It is interesting to note that sclerotia which developed on media containing high concentrations of coumarin (50–100 p.p.m.) were darker in colour and larger than the normal ones (Fig. 2). A hundred sclerotia grown on the control medium measured on an average 0·74 mm. while an equal number of

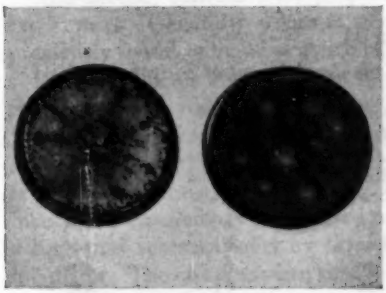


FIG. 1

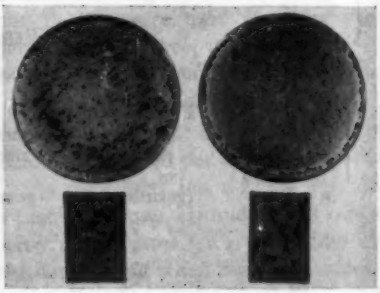


FIG. 2

FIG. 1. Abnormal development of cultures grown from sclerotia of very high viability on media containing 500 p.p.m. coumarin.

FIG. 2. Development of sclerotia and difference in their size on a medium containing 100 p.p.m. coumarin in comparison with control media.

Left: control. Right: medium containing inhibitor.

sclerotia grown on the medium containing 100 p.p.m. coumarin was 1·19 mm., a difference of 60·8 per cent. The mycelium growing on this medium does not develop its sclerotia in the usual way. While normally they are distributed all over the plate, under the influence of 100 p.p.m. coumarin they develop only on the walls of the petri-dish, so that they are not in contact with the substrate (Fig. 2). This increase in the size of the sclerotia, which is due to coumarin, persisted for another generation even when grown on inhibitor-free medium.

Sclerotia which developed on a substrate containing coumarin when planted on media without the inhibitor germinated faster than sclerotia from

control cultures. But if they were transferred to media containing one of the higher concentrations of coumarin their behaviour was similar to that of normal sclerotia (Fig. 3).

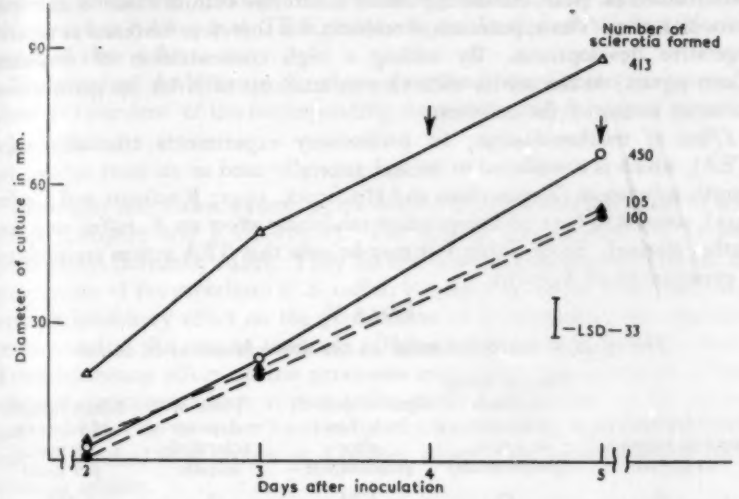


FIG. 3. The growth of mycelium from sclerotia formed on a medium containing coumarin in comparison to normal sclerotia, on control and coumarin containing medium. (L.S.D. calculated for $P = 0{\cdot}05$.)

Δ Mycelium from sclerotium formed on 100 p.p.m. coumarin germinating on normal medium: ▲ similar mycelium germinating on 100 p.p.m. coumarin: o mycelium from normal sclerotia inoculated on to normal medium: ● similar mycelium inoculated on to a medium containing 100 p.p.m. coumarin: ↓ first appearance of sclerotia on the culture.

TABLE IV

The effect of α-naphthylacetic acid on the development of S. rolfsii

Concentration of α-naphthylacetic acid in p.p.m.	Mean time in hours to reach diameter of 1 mm. (germination)	Rate of growth mm./hour after germination	Time in days to sclerotial initials	Mean number of sclerotia formed per plate
0	41	1·28	5	426
2	*46*	1·14	6	*350*
10	48	1·16	6	300
50	51	*0·88**	6	300
100	52	0·62*	6½	210
500		no growth		
L.S.D. at $P = 0{\cdot}05$	4	0·20	1½	41

* Compact, low, and dense mycelia

The effect of various concentrations of NAA on the cultures of *S. rolfsii* was investigated (Table IV).

The table clearly shows that NAA delayed germination and reduced sclerotium formation even at as low a concentration as 2 p.p.m. The rate of

growth of the mycelia was significantly lower at the higher concentrations. Compactness and density of mycelium at 50–100 p.p.m. were most pronounced during the first half of the growth period. It was noted that the first sclerotia were formed on NAA containing media before the culture reached its maximum diameter. The appearance of sclerotia was therefore hastened as regards vegetative development. By adding a high concentration of coumarin (1,000 p.p.m.) to the media with various amounts of NAA, no germination occurred in any of the cultures.

Effect of triethanolamine. In preliminary experiments triethanolamine (TEA), which is considered to be and generally used as an inert solvent for growth substances (Zimmerman and Hitchcock, 1942; Kaufman and Crafts, 1945), seemed to have an independent inhibiting effect on *S. rolfsii*, and was further studied. From Table V it may be seen that TEA acts as an inhibitor of germination of *S. rolfsii*.

TABLE V

The effect of triethanolamine on the development of S. rolfsii

Concentration of triethanolamine in p.p.m.	Time in hours to reach diameter of 1 mm. (germination)	Rate of growth mm./hour after germination	Time in days to sclerotial initials	Mean number of sclerotia formed per plate
0	39	1·39	5	388
120	46	1·39	5	378
240	51	1·32	5	360
480	56	1·32	5	339
960	80	1·28	7	230
1,980	158	1·35	10	190
3,840		no growth		
L.S.D. at $P = 0{\cdot}05$	5	0·15	1	27

It should be noted that in some series, where the viability of the mother sclerotia was not very high, concentrations over 1,000 p.p.m. of TEA in the medium were enough to inhibit germination of sclerotia completely.

Here, as with the other inhibitors described above, there was no effect on growth-rate of the cultures.

In order to determine whether the tardy development of the culture was actually due only to a delay in germination of the mother sclerotia, we inoculated mycelium of *S. rolfsii* on to the media containing high concentrations of TEA (3,840 and 7,680 p.p.m.) which completely inhibited germination of sclerotia. At the lower concentration, all the plates developed normally but for the total number of sclerotia which was, as expected, low. Only on some of the plates containing the highest concentration did the mycelia develop weakly, and no normal sclerotia were produced. No lag period appeared in either of the concentrations.

We were able to show that sclerotia, completely inhibited either by coumarin or by TEA, will germinate and produce normal cultures if transferred to control potato glucose agar media. The only holdover effect of the inhibitors

which could be detected was a decrease in the total number of sclerotia produced in comparison with the control. While a typical culture from a normal sclerotium produced 466 new sclerotia, those which were for 8 days on a medium containing 1,000 p.p.m. coumarin produced only 256 and other, which were on 7,680 p.p.m. TEA, produced 302. In all cases the first sclerotia appeared after 5 days.

The standard deviation in the above described experiments was generally about 5–7 per cent. of the corresponding mean value and in no case exceeded 9 per cent.

DISCUSSION

Coumarin and maleic hydrazide are known to be growth-inhibitors of seed plants (Leopold and Klein, 1951). The first is also an inhibitor of seed germination (Evenari, 1949). They do not have a pronounced effect on the growth-rate of the mycelium of *S. rolfsii*; but they, as well as TEA and NAA, have an inhibitory effect on the germination of the sclerotia; this was most pronounced in the case of coumarin. These substances also seem to have a direct inhibiting effect on the processes controlling the formation of new sclerotia, processes which appear to be partly independent of the normal growth. NAA on the other hand induced sclerotium development before the cultures reached their maximum diameter, which is the normal stage at which sclerotia appear.

The size of the sclerotia decreases as their number increases, if their nutrient supply remains the same (Lavee, 1955). In the presence of inhibitors the size of the sclerotia often increased as a result of a reduction in their number, and in the case of coumarin, the enlarging effect persisted for another generation. Coumarin seems to have a direct effect on *S. rolfsii* also because it causes the sclerotia to develop a darker colour than controls. The fungistatic effect of coumarin and MH on sclerotia of *S. rolfsii* accords well with their general activity as germination and growth inhibitors. Previous work (Lavee, 1955) showed that older sclerotia had lower viability. The effects of an inhibitor on these old sclerotia are more pronounced.

NAA caused inhibition of growth at higher concentrations (50–100 p.p.m.) and caused the mycelium to grow in a more compact and dense form. NAA hastened the development of sclerotia as related to the development in diameter of the culture.

TEA, which is often used as an inert solvent for growth substances (Zimmerman and Hitchcock, 1942; Kaufman and Crafts, 1945), was found to have a rather strong inhibiting effect on *S. rolfsii*, and has a fungistatic effect on the sclerotia.

LITERATURE CITED

Crowdy, S. H. (1948). Treatment of apple canker lesions with plant growth substances. *Nature*, **161**, 320–1.

Cursi, M. (1932). Studi su lo Sclerotium rolfsii. *Bull. R. Staz. Pat. Veg.* N.S. **11**, 306–73.

Davis, D., Dimond, A. E. (1953). Inducing disease resistance with plant growth regulators. *Phytopathology*, **43**, 137–50.

EVENARI, M. (1949). Germination inhibitors. *Bot. Rev.* **15**, 153–94.

KAUFMAN, P. B., CRAFTS, A. S. (1945). Responses of the rice plant to different formulations and methods of application of 2,4-D, MCP, and 2,4,5-T. *Hilgardia*, **24**, 411–53.

LAVEE, S. (1955). *Studies in the physiology and pathology of Sclerotium rolfsii (Sacc.) and apple resistance.* Master thesis, Hebrew University, Jerusalem.

LEOPOLD, A. C., KLEIN, W. H. (1951). Maleic hydrazide as an anti-auxin in plants. *Science*, **114**, 9–10.

NAKATA, K. (1927). Studies on Sclerotium rolfsii. Part IV (Japanese). Abstract Review Applied Mycology, **7**, 120.

PELEG, J. (PERLBERGER) (1945). Root rot in deciduous fruit trees caused by S. rolfsii and its control. *Hassadeh*, **25**, 467–70.

—— (1947). Orchard treatment to control root rot caused by S. rolfsii. *Hameshek Hackaklai*, **8**, 23.

ROSEN, H. R., SHAW, L. (1929). Studies of Sclerotium rolfsii, with special reference to the metabolic interchange between soil inhabitants. *Agric. Res.* **39**, 41–61.

SAMISH, R. M. (1954). Dormancy in woody plants. *Ann. Rev. Plant Physiol.* **5**, 183–204.

ZIMMERMAN, P. W., HITCHCOCK, A. E. (1942). Substituted phenoxy and benzoic acid growth substances and the relation of structure to physiological activity. *Contr. Boyce Thompson Inst.* **12**, 321–43.

Factors Affecting the Abscission of Reproductive Organs in Yellow Lupins (*Lupinus luteus* L.)

III. ENDOGENOUS GROWTH SUBSTANCES IN VIRUS-INFECTED AND HEALTHY PLANTS AND THEIR EFFECT ON ABSCISSION

R. F. M. VAN STEVENINCK

Crop Research Division, Department of Scientific and Industrial Research, Christchurch, New Zealand[1]

Received 1 November 1958

SUMMARY

Extracts of small and mature-size lupin pods yielded four substances affecting the growth of wheat-coleoptile sections: one acid promotor (*A*), two acid inhibitors (*B* and *X*), and one neutral inhibitor (*Y*).

Inhibitor *B* was extremely active, however, coleoptile sections showed no signs of toxic effects; they resumed growth at a rapid rate after rinsing them and adding β-indolylacetic acid (IAA) to the medium. 1 μg of IAA was required to counteract the effect of '*B*' extracted from 230 mg. of tissue. On an equal fresh weight basis the inhibiting action of '*B*' in lupin pods was 500–1,500 times more potent than that of 'inhibitor β' in etiolated pea seedlings.

Small pods of plants infected with pea-mosaic virus yielded 3 times the amount of '*A*' of healthy plants (equivalent to 1 μg. IAA and 0·3 μg. IAA per 25 g. of tissue respectively), and approximately $\frac{2}{3}$ the amount of '*B*'. Mature pods of virus-infected plants again yielded more '*A*', but also 2½ times more '*B*' than pods of healthy plants. Healthy pods yielded more '*Y*' than virus-infected pods, and there was no difference in '*X*'.

A lupin abscission test was developed and the effects of proximal and distal application of α-naphthyl acetic acid (NAA) are presented, and discussed with respect to results of other abscission tests.

'*A*' accelerated abscission when applied proximally, and delayed or prevented it when applied distally. '*B*' strongly accelerated abscission when applied in either way. A possible mechanism explaining the abscission-inducing effect of developing pods on later flowers is discussed in terms of the substances '*A*' and '*B*'. The partly prevented abscission observed on virus-infected plants was found to agree well with the proposed mechanism.

INTRODUCTION

WHEN a certain proportion of the flowers of yellow lupins (Var. Weiko II) were substituted with a wide range of auxins and growth inhibitors, it was shown that under normal conditions probably some other substance than auxin, possibly a growth inhibitor or anti-auxin, was involved in the initiation of flower abscission (Van Steveninck, 1958*a*).

Osborne's finding that an abscission accelerating factor other than auxin

[1] Present address: Department of Botany, King's College, London.

was produced in senescent leaves (1955) or in normal green leaves (1958) as a result of application of auxin added some support to the above hypothesis. Yet it was shown by several authors (Wright, 1956; Luckwill, 1957) that only auxins affected abscission, and natural inhibitors were shown to be without effect. However, in these cases the material used for assay of the growth substances (*Coleus*) was different from that of the plants under investigation (blackcurrant and apple respectively). There is increasing evidence that various plant species produce, and only respond to their own specific set of growth substances, and hence it should be a distinct advantage to use identical plant material for the assay as well as for the extraction of the growth substances.

It was previously shown that abscission is partly prevented on plants infected with pea mosaic virus (Van Steveninck 1957*a*). It was therefore decided to analyse the ether extracts of developing pods in healthy and infected plants and to compare their activity in the coleoptile straight growth-test and a lupin abscission test which was designed especially for this purpose.

MATERIALS AND METHODS

All experimental plants were grown in the greenhouse and virus inoculations were made at the developmental stage which would cause a maximum prevention of abscission (cf. Van Steveninck, 1957*a*). Pods were collected either 7 days after flowering, when they were very small (mean weight 135·5 mg. for virus infected and healthy plants), or 14 days after flowering, that is when they had almost reached mature-size (mean weight 1·4 and 1·6 g. for virus-infected and healthy plants respectively). They were frozen immediately on collection and kept at −20° C. until extractions were carried out.

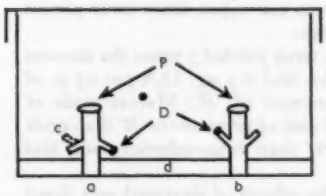


FIG. 1. A schematic representation of the lupin abscission test; (*a*) 'Explants' apical end down; (*b*) basal end down; (*c*) position of pedicels after 24 hours; (*d*) 2 per cent. agar; *P*—proximal, *D*—distal application.

The frozen pods were cut to uniform-sized blocks, and extracted with approximately three times their volume of peroxide-free ether for 16 hours at +1° C. Purification of extracts, chromatography, and coleoptile straight growth-test were the same as described previously (Van Steveninck, 1958*b*).

The lupin abscission test. The lupin abscission test essentially is an adaptation of Addicott's abscission test (Addicott *et al*, 1949). The 'explants' consisted of a 2-cm. section of the main flower stalk with the 5 pedicels half-way, and the flowers removed. Directly after cutting 10 'explants' were placed upright in 5 mm. thickness of 2 per cent. agar which was allowed to set in 9 cm. crystallizing dishes, covered by a petri-dish lid. At least 2 dishes were used for each treatment. Substances under investigation could either be applied to one of two proximal sides (physiological base or apex, cf. Fig. 1), or to the

distal side of the abscission zone. The substances were applied either through the intermediary of punched filter-paper disks (directly from chromatograms, or impregnated with a known quantity of purified extract), which were glued to the 'explants' with 15 per cent. gelatine kept at 60° C., or applications were made as a lanolin paste (1 mg. per pedicel). Checks on abscission were made at 9 a.m., 4 p.m., and 11 p.m.

Simultaneous tests of all whorls of whole inflorescences have shown that little or no difference existed in the time required to complete the abscission process once the 'explants' were cut. However, for most of the following work 'explants' were taken from the 2nd–5th whorls.

Contamination was avoided as much as possible by carrying out all the work in a transfer chamber in a specially appointed constant-temperature room kept at +27° C.

RESULTS

Growth substances in virus-infected and in healthy pods. Four different substances were found to affect the growth of wheat coleoptile sections (Fig. 2).

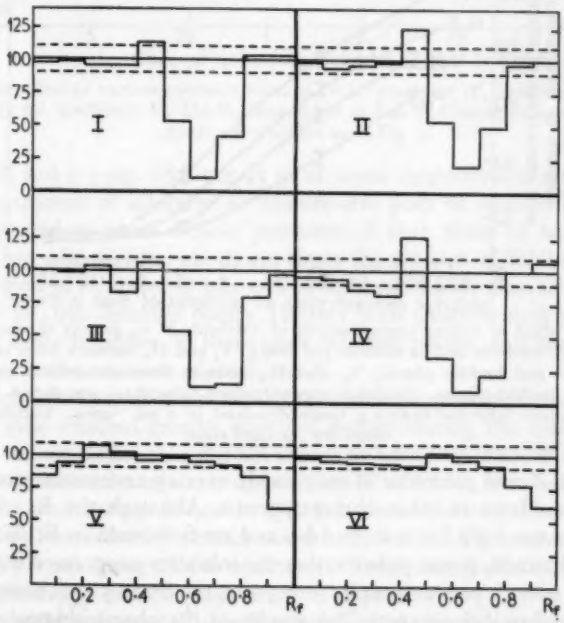


FIG. 2. The response of wheat coleoptile sections to chromatogram strips prepared from 10 g. samples of lupin pods. Solvent: *iso*propanol, ammonia 25 per cent., water = 8:1:1. I and II. Acid fractions of small pods. III and IV. Acid fractions of mature-size pods. V and VI. Neutral fractions of mature-size pods. I, III, V healthy pods; II, IV, VI virus-infected pods. Growth has been plotted as a percentage of the growth of control sections; the broken line represents the level at which differences were significant ($P = 0{\cdot}05$).

The mean growth of control sections was 7·80–8·65 mm. over a period of 17–18 hours.

1. A neutral inhibitor (*Y*) with an R_f value of about 0·9 and showing a strong fluorescence in U.V. light.
2. A weak, acid inhibitor (*X*) with $R_f = 0{\cdot}3–0{\cdot}4$; it was detected in mature-size pods only.
3. An acid growth promotor (*A*) with $R_f = 0{\cdot}4–0{\cdot}5$.
4. A very potent inhibitor (*B*) with $R_f = 0{\cdot}55–0{\cdot}75$.

The area of inhibitor *B* always gave a distinct Ehrlich reaction (dark, rather dirty pink spot). Further work with the partly purified inhibitor extract and the use of other solvent systems, however, showed that the substance responsible for the Ehrlich reaction had no effect on straight growth. Although inhibitor *B* caused up to 90 per cent. inhibition of coleoptile extension, the sections did not change colour, remained fully turgid, and were capable of resuming growth at a rapid rate after rinsing them in distilled water and adding β-indolyl acetic acid (IAA) to the medium.

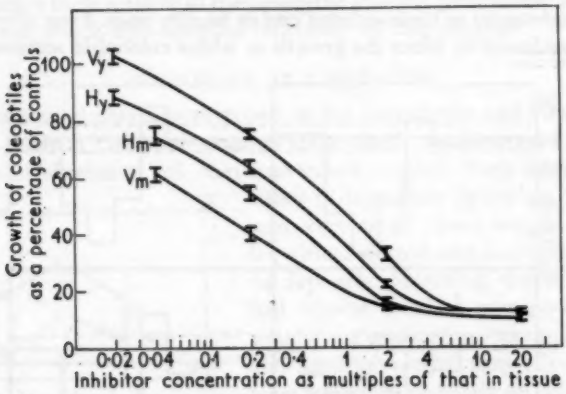


FIG. 3. The effect of various concentrations of 'inhibitor *B*' on growth of wheat coleoptile sections (1 ml. medium and 10 sections per tube). V_y and H_y, extracts from small pods of virus-infected and healthy plants; V_m and H_m extracts from mature-size pods of virus infected and healthy plants. Inhibitor concentrations (abscissae) are shown as 'normalities': 1 N. means inhibitor from 1 g. tissue dissolved in 1 ml. water. Vertical lines, two times the standard error.

Inhibitor *X* and promotor *A* may partly overlap and sometimes cancelled each others effects in other chromatograms. Although the R_f value of the promotor is too high for it to be IAA and no Salkowski or Ehrlich reaction could be obtained, it was possible that the inhibitor suppressed the principal area of the growth promotor at $R_f = 0{\cdot}3–0{\cdot}4$, the 0·4–0·5 area being the outer part of a rather diffuse spot. The results of the abscission test seemed to support this idea.

The position of the four growth substances was determined by bioassay for a good many chromatograms, and a close correlation with the position of the fluorescent spots made it possible to cut out the appropriate areas for each of the growth substances. These strips were eluted with three consecutive lots

of 30 ml. ether, shaken for 2 hours, the eluate concentrated, the concentrate applied to paper strips in a wide range of quantities, and the strips tested with the coleoptile straight growth-test of which the results are shown in Figs. 3 and 4. It was found that small pods of virus infected plants yielded approximately 3 times the amount of promotor *A* of healthy plants (equivalence of

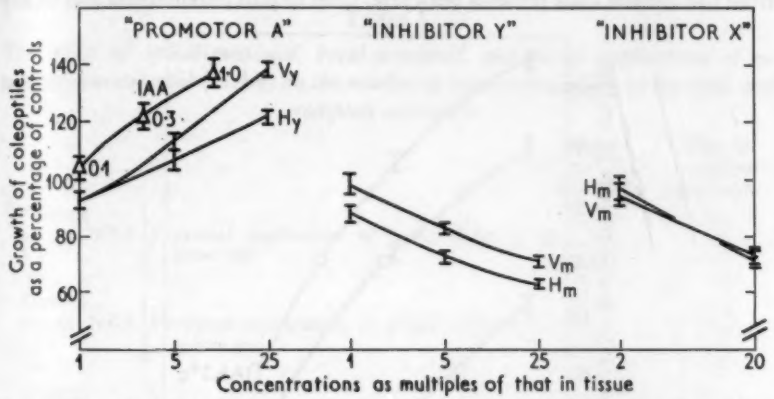


FIG. 4. The effect of various concentrations of IAA, 'promotor *A*', 'inhibitor *X*', and 'inhibitor *Y*' (1 ml. medium). V_y, H_y, V_m, and H_m as in Fig. 3. Concentrations and standard errors are recorded as in Fig. 3.

1 μg. IAA and 0·3 μg. IAA per 25 g. of tissue respectively), and about 0·4 times the amount of inhibitor *B*. Mature-size pods of virus-infected plants again appeared to contain more promotor *A* than those of healthy plants (Fig. 2), but also yielded about 2·5 times the amount of inhibitor *B*. The concentration of inhibitor *B* was greater in mature pods than in small pods, especially in virus infected plants. Healthy pods (mature-size) yielded considerably more inhibitor *Y* than virus infected plants, and there were no differences in the amounts of inhibitor *X*.

Although inhibitor *B* could cause the maximum possible inhibition of 90 per cent. (the residual growth occurred mainly during the 2-hour soaking period of the cut sections), it did not appear to have a toxic effect. Interactions between various concentrations of inhibitor *B* and IAA were measured and some of the results are presented in Fig. 5. It shows that the effect of inhibitor *B* can be counteracted by certain concentrations of IAA, amounts of 1 and 3 μg. IAA per ml. being able to counteract the inhibiting effect of inhibitor *B* extracted from 230 and 700 mg. of tissue respectively.

The lupin abscission test. Table I is included to give an impression of how the test behaves towards known quantities of α-naphthylacetic acid (NAA). At once one can observe that proximal applications accelerated abscission quite strongly when they were applied to the physiological base of the 'explant'. Apical applications were much less effective except with high concentrations of auxin, irrespective of whether 'explants' were placed

upright or not. These results indicated that auxin in the lupin flower stalk is predominantly transported in apical direction, and confirm the results of previous investigations on whole plants (Van Steveninck, 1958*a*).

Anatomical investigations showed that the first signs of differentiation at 48 hours after cutting, i.e. a gradual lignification of the cell walls proceeding from the cortical cells towards pericycle, then to pith parenchyma and finally

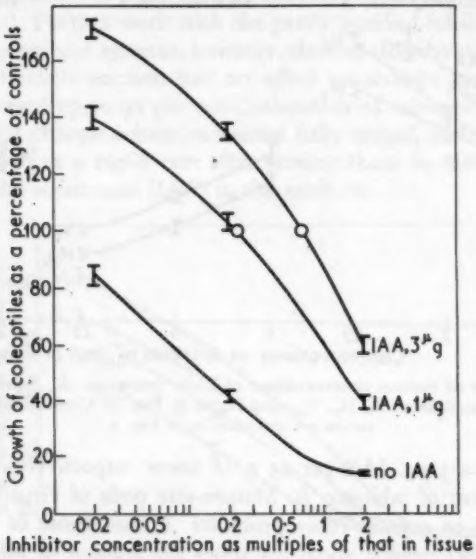


FIG. 5. The effect of various amounts of IAA in alleviating the growth inhibition caused by various amounts of 'inhibitor *B*'. Concentrations and standard errors are recorded as in Fig. 3.

to the phloem, started approximately 24 hours earlier, and was speeded up by approximately 20 hours with a proximal application of NAA at 0·1 per cent.

With distal application lanolin preparations of NAA delayed or completely prevented abscission. Pedicels became twice as long as the control ones and also increased considerably in diameter. The same happens to pedicels *in situ* when they carry developing fruits, however, in contrast to the pedicels *in situ* no additional vascular tissue was laid down.

Table I shows further that very low concentrations of NAA (10^{-7} and 10^{-6} M.) added to the agar medium (proximal application) delayed abscission and promoted it at 10^{-3} M. These results in lupins are essentially different from those of Leopold and his co-workers (1955, 1958), who obtained an acceleration of abscission from 10^{-7} M. to 10^{-4} M. NAA and a total prevention at 10^{-3} M. in their bean abscission test. Both Leopold's and my results therefore fail to support Addicott's gradient theory (1955), although results with intermediate concentrations (10^{-5} M., 10^{-4} M.) are in agreement. Further, it is of interest to note that unlike the logarithmic concentration,

elongation relationship of growth of coleoptile and other tissues, abscission responses on whole plants only occur with concentrations between 10^{-5} and 10^{-4} M. (1·9 and 19 p.p.m.). Leopold's results with 10^{-3} M. NAA are almost certainly due to toxicity, and it is possible that the very low concentrations in our test acted through other channels which indirectly influenced abscission.

TABLE I

The effect of apical–proximal, basal–proximal, and distal applications of α-naphthyleacetic acid (NAA) on the number of hours required for 50 per cent. and complete abscission

		Hrs. to 50 per cent. abscission	Hrs. to complete abscission
Control	Proximal application of paper disks at the basal end	84·0	114
0·1 μg. NAA		73·5	97
1 ,,		68·4	97
10 ,,		60·9	90
Control	Proximal application of paper disks at the apical end	86·3	114
0·1 μg. NAA		91·2	114
1 ,,		83·5	114
10 ,,		74·3	97
Control	Proximal application at basal end by adding NAA to agar medium	84·6	117
NAA, 10^{-3} M		61·6	83
,, 10^{-4} ,,		68·1	99
,, 10^{-5} ,,		79·9	120
,, 10^{-6} ,,		92·2	117
,, 10^{-7} ,,		94·5	164
Control	Distal application of NAA in lanolin	114·5	170
NAA, 0·001%		134·1	170
,, 0·01%		185·8	∞
,, 0·1%		∞	∞

The effect of lupin growth substances on the abscission test. Again the four growth substances were extracted separately from their corresponding areas on the chromatograms; the purified extracts were reduced in volume and applied to paper disks in quantities equivalent to 5, 1, 0·2, and 0·04 g. of fresh tissue, or they were mixed with small quantities of lanolin paste (with inhibitor *B* an amount equivalent to 250 mg. of fresh tissue per mg. of lanolin, and for the other three substances the equivalent of 500 mg. of fresh tissue per mg. of lanolin).

Table II shows that 'inhibitor *B*', equivalent to 5 g. fresh tissue per 'explant', applied *proximally* stimulated abscission quite strongly, 1 g. per explant or less had little or no effect. 'Promotor *A*' (5 g. per 'explant') applied proximally had a slightly smaller but qualitatively similar effect to 'inhibitor *B*'. Again 1 g. per 'explant' had little or no effect. 'Inhibitor *X*' applied proximally also promoted abscission. 'Inhibitor *Y*' did not seem to have any significant effect on abscission.

With *distal* application, 'inhibitor *B*' accelerated abscission quite strongly when applied as 1 mg. of lanolin emulsion equivalent to 250 mg. of fresh tissue

per pedicel. This is twice the weight of a small pod; however, with all the above treatments one can expect only a fraction of the applied growth substance to enter the 'explant'. Both 'promotor *A*' and 'inhibitor *X*' delayed abscission considerably when applied distally. It was not possible to obtain significant differences between abscission responses when equivalent amounts of promotor or inhibitor from virus-infected and healthy plants were applied to the explants.

TABLE II

The effect of the four endogenous growth substances in lupins on the number of hours required for 50 per cent. and complete abscission respectively

		Hrs. to 50 per cent. abscission	Hrs. to complete abscission
Control	Proximal application	81·4	115
'Inhibitor *B*' eq 5 g.		64·5	84·5
,, ,, 1 ,,		81·9	97·5
'Promotor *A*' eq 5 ,,		74·9	93·5
,, ,, 1 ,,		81·1	100
Control	Proximal application	91·5	112
'Inhibitor *X*' eq 5 ,,		83·6	98
,, ,, 1 ,,		89·6	112
'Inhibitor *Y*' eq 5 ,,		86·0	112
,, ,, 1 ,,		88·2	112
Control	Distil application	87·2	134
'Inhibitor *B*' eq 250 mg.		72·0	89
'Promotor *A*' eq 500 ,,		130·5	∞
'Inhibitor *X*' eq 500 ,,		138·6	∞
'Inhibitor *Y*' eq 500 ,,		89·7	140

An observation of considerable interest was that distal application of 'promotor *A*' increased the geotropic curvature of the pedicels on explants, placed with their basal end up. 'Inhibitor *B*', on the other hand, prevented this geotropic curvature entirely. This seems to be correlated well with the fact that young virus infected pods assume a much more acute angle relative to the flower stalk than pods of healthy plants, and later again when reaching mature size their position is more horizontal than healthy pods.

DISCUSSION

It was shown that abscission in lupins is controlled by the presence of an auxin ('promotor *A*') and one or possibly two growth inhibitors ('inhibitors *B* and *X*'). It seems possible now to explain much more intelligently the fact that the first developing pods have an abscission inducing effect on later flowers (Van Steveninck, 1957*b*). The flowers on the basal whorls, which are first to open, produce small pods, and these are likely to develop because both substances being produced distally to the abscission zone '*A*' and '*X*' will counteract the abscission accelerating effect of '*B*', however, once all three substances arrive at the flower stalk, they are preferentially translocated in the apical direction and being proximal to the abscission zones they all co-operate in accelerating abscission of further flowers.

The fact that small pods of virus-infected plants produce more *A* and less *B* seems to be correlated well with the previous observation that flower abscission on infected plants is reduced to a considerable extent (Van Steveninck 1957*a*). In addition, the orientation of the pods relative to the flower stalk during the course of their development correlated very well with the changes in relative concentrations of *A* and *B* observed in virus-infected and healthy plants.

It was rather unexpected that both 'promotor *A*' and 'inhibitor *X*' had the same effect on abscission; it appears more likely that '*X*' by itself had no effect on abscission, and was obscuring part of the activity of '*A*' in the straight growth test, and hence the identical effect of '*A*' and '*X*' in the abscission test may have been due to '*A*' only. Likewise the higher concentration of 'promotor *A*' in the virus infected pods may have been due to a lower concentration of 'inhibitor *X*' in the virus-infected tissues. For instance, it was not possible to obtain a significant difference in abscission responses when equivalent amounts of promotor from virus-infected and healthy pods were applied to the explants.

It seems rather difficult to explain the extremely strong growth-inhibiting effect of 'inhibitor *B*' relative to the rather weak auxin effect of 'promotor *A*' in a tissue of which the cells are elongating very rapidly. For instance, the activity of 'inhibitor *B*' in lupin pods was 500–1,500 times greater than the activity of 'inhibitor β' from an equal amount of etiolated pea seedlings extracted under identical conditions. Of course, the extraction technique may have been such as to give us a biased picture of the relative importance of the growth substances, and there is also a possibility that 'inhibitor *B*' inhibits cell-elogation in wheat coleoptiles much more than in lupin tissues. Yet, the paradox remains that the virus-infected pods grew less rapidly even though more auxin and less inhibitor was found to be present in healthy pods. But then again one can visualize the virus infection to have numerous effects on processes which may determine growth. At this juncture it is interesting to recall the results of an earlier experiment (Van Steveninck, 1958*a*) in which the internodal elongation between whorls of the flower stalk was found to be inhibited by a low concentration of auxin (NAA or IAA) and to be promoted by the auxin-antagonist 2:3:5-tri-iodobenzoic acid. Clearly, many experiments remain to be done to find out more about the relative and physiological significance of the four lupin growth substances.

Acknowledgement

The author would like to thank Miss Margaret Hampton for her capable technical assistance.

Literature Cited

Addicott, F. T., Lynch, R. S., Livingston, G. A., and Hunter, J. K. (1949). A method for the study of foliar abscission *in vitro*. *Plant Physiol.* **24**, 537–9.

—— —— and Carns, H. R. (1955). Auxin gradient theory of abscission regulation. *Science*, **121**, 644–5.

BIGGS, R. H., and LEOPOLD, A. C. (1958). The two-phase action of auxin on abscission. *Amer. J. Bot.* **45**, 547–51.

GAUR, B. K., and LEOPOLD, A. C. (1955). The promotion of abscission by auxin. *Plant Physiol.* **30**, 487–90.

LUCKWILL, L. C. (1957). Studies of fruit development in relation to plant hormones. IV. Acidic auxins and growth inhibitors in leaves and fruits of the apple. *J. Hort. Sci.* **32**, 18–33.

OSBORNE, D. J. (1955). Acceleration of abscission by a factor produced in senescent leaves. *Nature*, **176**, 1161–3.

—— (1958). The role of 2:4:5-T butyl ester in the control of leaf abscission in some tropical woody species. *Trop. Agric.* **35**, 145–58.

VAN STEVENINCK, R. F. M. (1957*a*). Influence of pea-mosaic virus on the reproductive capacity of yellow lupine. *Bot. Gaz.* **119**, 63–70.

—— (1957*b*). Factors affecting the abscission of reproductive organs in yellow lupins (*Lupinus luteus* L.). I. The effect of different patterns of flower removal. *J. Exp. Bot.* **8**, 373–81.

—— (1958*a*). Factors affecting the abscission of reproductive organs in yellow lupins (*Lupinus luteus* L.). II. The effect of growth substances, defoliation, and removal of lateral growth. Ibid. **9**, 372–83.

—— (1958*b*). Straight-growth inhibitors in glucose. *Nature*, **182**, 950–1.

WRIGHT, S. T. C. (1956). Studies of fruit development in relation to plant hormones. III. Auxins in relation to fruit morphogenesis and fruit drop in the blackcurrant. *Ribes nigrum*. *J. Hort. Sci.* **31**, 196–211.

An Endogenous Rhythm in the Rate of Carbon Dioxide Output of Bryophyllum[1]

I. SOME PRELIMINARY EXPERIMENTS

M. B. WILKINS

Department of Botany, King's College, London

Received 10 December 1958

SUMMARY

A technique is described for recording automatically, with the aid of an infra-red gas analyser, the rate of CO_2 output or absorption by plant material under controlled conditions.

An examination of the rate of CO_2 output by excised leaves of 16 species of succulent plants in darkness and in a CO_2-free atmosphere revealed clearly defined rhythms in only *Bryophyllum fedtschenkoi*, *B. daigremontianum*, and *B. calycinum* (*pinnatum*).

Further investigation of the rhythm in leaves of *B. fedtschenkoi* revealed that: (1) daylength has no effect upon the period of the rhythm in subsequent darkness, the phase being set at the time the lights are extinguished; (2) normal air suppresses the rhythm; (3) removal of the epidermis and cutting the mesophyll into pieces 1 cm^2 does not affect either the phase or period of the rhythm; (4) continuous illumination at an intensity of 3,000 lux inhibits the rhythm which restarts when the lights are extinguished. The phase of the rhythm can be set at any time of day according to the time at which the lights are extinguished. The time which elapses between the onset of darkness and the first peak decreases as the length of the light treatment is increased.

The endogenous nature of the rhythm is fully established. The results are compared with those of other researches.

INTRODUCTION

THE occurrence of a rhythm in the rate of CO_2 output of *Avena sativa* coleoptiles when they are transferred from red light to darkness has been described by Ball, Dyke, and Wilkins (1957). The relatively short life, rapid growth, and early emergence of the leaf made cereal coleoptiles unsuitable for the study of endogenous rhythms in CO_2 metabolism. The need for a non-growing or slowly growing tissue with sufficient food reserves to sustain itself for several days in darkness focused attention upon the excised leaves of succulent plants and a number of species were examined for rhythmicity. Of these *B. fedtschenkoi* proved to be particularly suitable and this series of papers comprise the results of an investigation of the effect of variation in external conditions upon the endogenous rhythm in this species.

[1] This work formed part of a thesis presented to the University of London for the degree of Ph.D.

The CO_2 metabolism of plant tissues has been used in several studies of 'biological clocks'. Excised leaves of *Helianthus annuus* show a 24-hour rhythm in their rate of 'respiration' (Spoehr and McGee, 1923) but the authors have not drawn attention to this fact. Neeb (1952), Pirson and Döring (1952), Pirson *et al.* (1954), and Schön (1955) have studied endogenous rhythms in the rate of CO_2 output and oxygen uptake in algae, especially in *Hydrodictyon reticulatum*. A rather poorly defined rhythm was observed in the rate of CO_2 output of excised leaves of *Kalanchoë blossfeldiana* in darkness and in an atmosphere initially free of CO_2 (Schmitz, 1951). The phase of the rhythm was easily modified by variation in external conditions and was set by the change from light to darkness, the first peak occurring 18 hours after the change at 25° C. Gregory, Spear, and Thimann (1954) did not detect a rhythm in the rate of CO_2 output of whole plants of *K. blossfeldiana* in darkness and in a stream of normal air. The plants fixed CO_2 during the first 16 hours of darkness and then gave out the gas at the constant rate for 32 hours. Nuernbergk (1955), studying the rate of CO_2 metabolism of plants under continuous illumination in normal air at 20–23° C., observed 24-hour rhythms in *Bryophyllum tubiflorum*, *B. daigremontianum*, and *Aloë arborescens*. In *Kalanchoë blossfeldiana* a rhythm was only just noticeable. Armin, Prinz zur Lippe (1956) detected a 24-hour rhythm in the rate of CO_2 output of primary leaves of *Phaseolus multiflorus* in darkness. The phase was determined by several factors, the chief being the ratio of the previous alternations of light and darkness. The longer the exposure to light immediately preceding the change to continuous darkness the shorter the time from that change to the first peak.

MATERIAL AND METHODS

Material. The succulent plants used in these experiments were available in the departmental garden. A plant of *Bryophyllum fedtschenkoi* was obtained some years ago from the Royal Botanic Gardens, Kew, and all leaves used in this research were excised from cuttings of the original plant. The size and degree of succulence of leaves of this species vary according to the day-length treatment to which they are subjected. Small very thick leaves were obtained by severely pruning and completely defoliating 1-year-old plants and subjecting them to short day illumination from 8 a.m. until 4 p.m. Leaves were used when they were approximately 2–3 months old.

Experimental methods. The arrangement of the apparatus is shown in Fig. 1. The apparatus was distributed between two rooms, a small laboratory and a constant-temperature dark room. A Grubb-Parsons infra-red gas analyser (type S.B. 1), having a condenser sensitive only to CO_2 and fitted with fluorite windows, received a power supply from a Grubb-Parsons A.C. voltage stabilizer (type A). Infra-red radiation, provided by heaters H_1 and H_2, passed through the absorption tubes *A* and *B* and was measured by the detecting condenser *D*. The presence of CO_2 in the sample tube (*B*) resulted in unequal intensities of radiation arriving at either side of the condenser

thus setting up a potential which was amplified and recorded on a specially modified Philips valve voltmeter. The meter reading was proportional to the

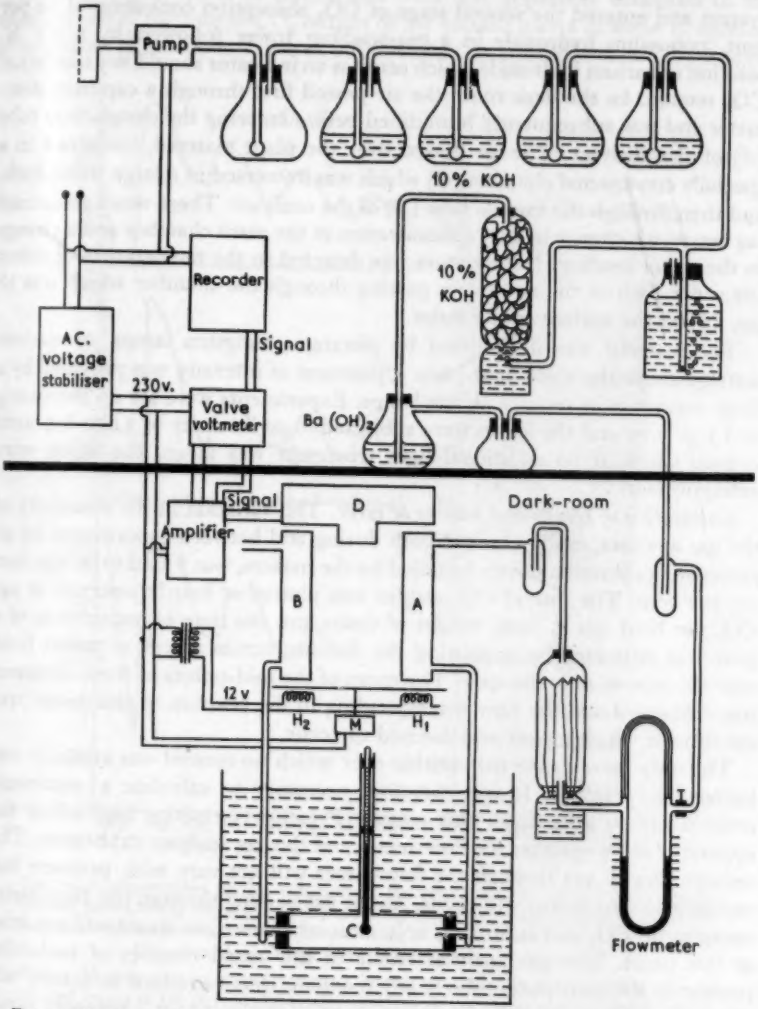


FIG. 1. Diagram to show the arrangement of the apparatus. For description see text.

concentration of CO_2 in the sample tube and was plotted automatically against time on the chart of an Elliott recorder (type 230).

Air, drawn from outside the laboratory by a 'Leon' twin cylinder synchronous pump, was filtered through cotton-wool and passed through several flasks containing 10 per cent. potassium hydroxide. A 2-mm. capillary

formed the outlet from the final flask; this provided a steady flow of air. The air then passed to a device which maintained a constant pressure in the system and entered the second stage of CO_2 absorption consisting of 10 per cent. potassium hydroxide in a gas-washing tower followed by a 0·1 N. solution of barium hydroxide which acted as an indicator should any undesired CO_2 remain. In the dark room the air passed first through a capillary flow-meter and was subsequently humidified before entering the comparison tube (*A*) of the analyser. The air flowed over the plant material, contained in a specially constructed chamber (*C*) which was immersed in a large water-bath, and then through the sample tube (*B*) of the analyser. There was a 20-second lag between a change in CO_2 concentration in the plant chamber and a change in the meter reading. No variation was detected in the temperature of either the water bath or the air stream passing through the chamber which was 18 cm. below the surface of the water.

The material was illuminated by placing 4 tungsten lamps of various wattage above the water-bath; fine adjustment of intensity was provided by a large resistance in series with the lamps. Experiments were set up between 3 and 3.30 p.m. and the leaves were subjected to an intensity of 3,000 lux until 4 p.m. when, if no additional light treatment was given, the lights were extinguished.

Calculation of results and sources of error. The variation in the sensitivity of the gas analyser, easily checked both during and between experiments by an automatic calibration device installed by the makers, was found to be less than 0·5 per cent. The rate of CO_2 output was plotted at hourly intervals in μg. CO_2 per hour per g. fresh weight of tissue and the time of occurrence of a peak was estimated by measuring the distance across a peak at points from near the base to near the apex. The mean of the mid-points of these distances was calculated and the time corresponding to the position of this mean was the time at which a peak was deemed to occur.

The only known external variable over which no control was available was barometric pressure. It was therefore necessary to calculate a maximum error likely to arise from this source. Pressure variations may affect the apparatus at two points: the flow-meter and the gas analyser calibration. The volume flow of gas through the flow-meter will not vary with pressure but variations in mass flow will occur. As the gas passing through the flow-meter contains no CO_2 and is, in fact, only an inert carrier gas errors will not arise at this point. The gas analyser reacts to the actual number of molecules present in the absorption tube at any moment, thus variations in density will affect the calibration. The maximum error of any point for a pressure variation of 2 inches will not exceed 0·8 per cent. The variation observed in the rate of CO_2 output of the plant material was so great as to make this error insignificant. Moreover, barograph fluctuations bore no relation to the fluctuations in the rate of CO_2 output exhibited by the plant material. A more complete description of the methods involved in this research is given by Wilkins (1958).

EXPERIMENTAL RESULTS

The initial experiments carried out with this apparatus consisted of an examination of the rate of CO_2 output of the excised leaves of some 16 species of succulent plants in darkness at 26° C. and in an atmosphere initially free of

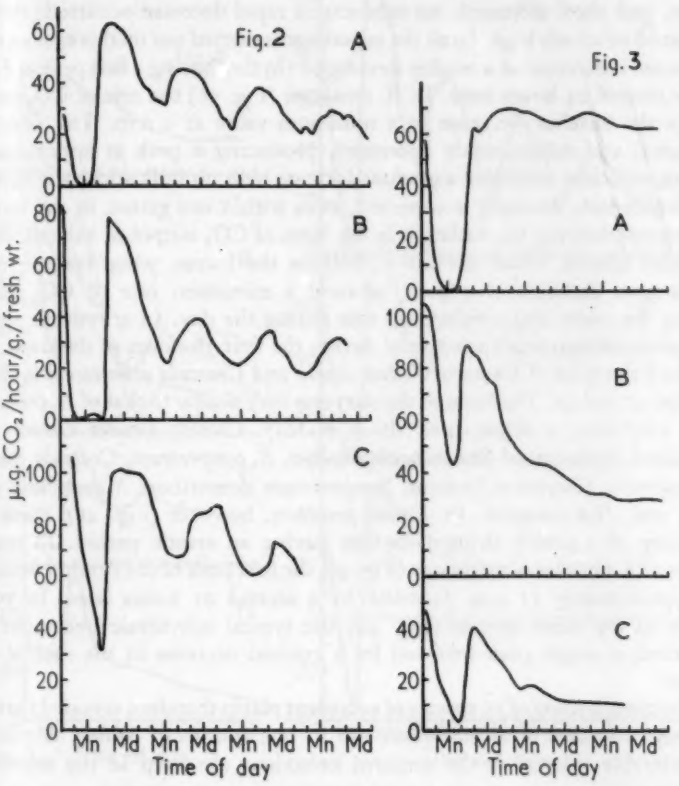


FIG. 2. A comparison of the variation in the rates of CO_2 output with time of excised leaves of (A) *Bryophyllum fedtschenkoi*, (B) *B. daigremontianum*, and (C) *B. calycinum* in continuous darkness commencing at 4 p.m. at 26° C. and in an initially CO_2-free atmosphere. Mn = MIDNIGHT. Md = MIDDAY.

FIG. 3. A comparison of the variation in the rates of CO_2 output with time of excised leaves of (A) *Bryophyllum tubiflorum*, (B) *B. crenatum*, and (C) *Sedum stahlli* in continuous darkness commencing at 4 p.m. at 26° C. and in an initially CO_2-free atmosphere.

CO_2. Five species of *Bryophyllum* were examined in this way. In *B. fedtschenkoi* (Fig. 2A) the high initial rate gradually decreased, reaching a minimum value at midnight. The rate then increased rapidly for 4–6 hours, levelled off, and then decreased, thus producing a peak at approximately 11 a.m. The rate

of output reached a second minimum value at about midnight and subsequently increased. The fluctuations persisted with a period of approximately 22 hours. Closely similar results were obtained using excised leaves of *B. daigremontianum* and *B. calycinum* (Figs. 2B and 2C). In *B. tubiflorum*, however (Fig. 3A), although the initial rate decreased to a minimum at about 1 a.m. and then increased, no subsequent rapid decrease occurred; the rate remained relatively high. In all the experiments carried out on this species there was some indication of a weakly developed rhythm having a first peak at 8 p.m. and a second 24 hours later. In *B. crenatum* (Fig. 3B) the rate of CO_2 output shows the familiar decrease to a minimum value at 1 a.m. The rate then increased and subsequently decreased, producing a peak at approximately 10 a.m. and then exhibited a gradual decrease with no indication of a rhythm.

Considerable diversity is observed, even within one genus, in the form of the curves showing the variation in the rates of CO_2 output of excised leaves. In three species which exhibited rhythms the leaves, when transferred to continuous darkness at 4 p.m., showed a minimum rate of CO_2 output during the night and a maximum rate during the day. In arhythmic species this phenomenon was shown only during the first 36 hours of darkness.

The behaviour of leaves of *Sedum stahlii* and *Crassula arborescens* is shown in Figs. 3C and 4A. The form of the curves is very similar to that of *B. crenatum*, each exhibiting a single peak about midday. Closely similar curves were produced by leaves of *Sedum pachyphyllum*, *S. compressum*, *Crassula pseudolycopodiodes*, *Cotyledon farinosa*, *Sempervivum domesticum*, *Mammilaria elongata*, and *Aloë varegata*. In *Sedum prealtum*, however (Fig. 4B), there was evidence of a poorly defined rhythm having an erratic period. In mature leaves of *Kalanchoë blossfeldiana* (Fig. 4C) the first peak of the rhythm occurred at approximately 11 a.m. followed by a second 21 hours later. In young leaves of the same species (Fig. 4D) the typical arhythmic behaviour was observed, a single peak followed by a gradual decrease in the rate of CO_2 output.

The examination of 16 species of succulent plants therefore revealed rhythms in only 3 species of *Bryophyllum* and in one species of *Sedum*. A point of considerable interest is the uniform behaviour observed in the arhythmic species.

The leaf size and perennial habit of *B. fedtschenkoi* made this species a convenient experimental material and an extensive investigation was made of the effect of variation in external conditions on the phase and period of the rhythm of CO_2 output of excised leaves of this species.

The effect of previous daylength treatment

(1) *Short days.* Whole leaves, excised from a plant which had been subjected to short-day illumination from 8 a.m. until 4 p.m., were maintained in darkness at a temperature of 26° C. for 116 hours commencing at 4 p.m. (Fig. 5A). The result is closely similar to that of the experiment shown in Fig. 2A which was carried out under identical conditions. The first peak of the rhythm

occurred at 11 a.m., 19 hours after the commencement of darkness. The rate then continued to fluctuate, exhibiting peaks at approximately 22-hour intervals. The period of the rhythm at 26° C., calculated from the experiments

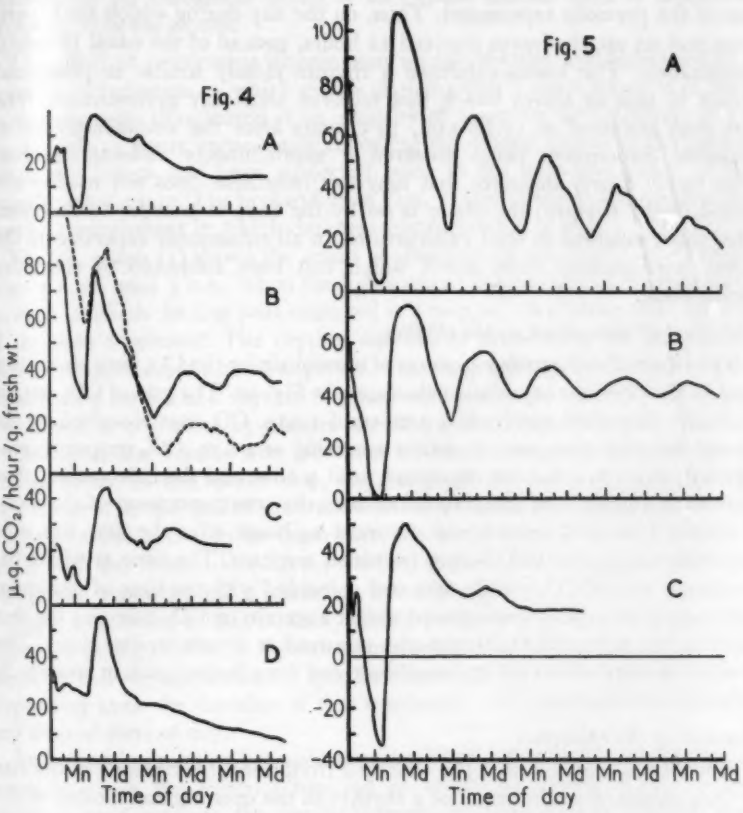


FIG. 4. A comparison of the variation in the rates of CO_2 output with time of excised leaves of (A) *Crassula arborescens*, (B) *Sedum prealtum*, (C) *Kalanchoë blossfeldiana* (mature), and (D) *K. blossfeldiana* (young) in darkness, at 26° C. and in an initially CO_2-free atmosphere.

FIG. 5. (A) Shows the rhythm in the rate of CO_2 output of excised leaves of *B. fedtschenkoi* in continuous darkness commencing at 4 p.m. at 26° C. and in an initially CO_2-free atmosphere following short (8 hrs.) day illumination. (B) Shows the course of the rhythm under identical conditions but following long (16 hrs.) day illumination. (C) Shows the course of the rhythm in darkness commencing at 4 p.m. at 26° C. but in a stream of normal air.

shown in Figs. 5A and 2A and from one further experiment not published, was $22{\cdot}4 \pm 0{\cdot}4$ hours. It is clear from Fig. 5A that the amplitude of successive peaks decreases with time in darkness and at a constant temperature.

(2) *Long days.* Fig. 5B shows the course of the rhythm in whole leaves, excised from a plant that had been subjected to long-day illumination from 4 a.m. until 8 p.m., in continuous darkness and at 26° C. The leaves were placed in darkness at 4 p.m. in order that the result might be comparable to that of the previous experiment. Thus, on the day during which the experiment was set up, the leaves received 12 hours, instead of the usual 16 hours, illumination. The leaves exhibited a rhythm closely similar in phase and period to that of leaves which had received short-day pretreatment. The first peak occurred at 11.30 a.m., 19·5 hours after the commencement of darkness. Subsequent peaks occurred at approximately 22-hour intervals. This result clearly indicates that long-day treatment does not modify the period of the rhythm; the phase is set by the time of transfer to darkness. This was confirmed in later experiments. In all subsequent experiments the leaves were excised from plants which had been subjected to short-day illumination.

The effect of normal air on the rhythm

The effect of substituting a stream of normal air for the CO_2-free air-stream used in the previous experiments is shown in Fig. 5C. The rate of CO_2 output gradually decreased and from 9 p.m. until 4 a.m. CO_2 was absorbed by the leaves, the maximum rate of uptake occurring at 2 a.m. CO_2 output recommenced at 4 a.m., the rate increased until 9 a.m. and then decreased, thus producing a peak at 11 a.m., 19 hours after the commencement of darkness. A weakly developed second peak occurred 24 hours after the first, but subsequently the rate of CO_2 output remained constant. The time at which the maximum rate of CO_2 uptake occurred coincided with the time of minimum CO_2 output in experiments carried out in a stream of CO_2-free air; the first peaks in the rates of CO_2 output also occurred at closely similar times. The rhythm persists with a greater amplitude and for a longer time in an initially CO_2-free atmosphere.

Removal of the epidermes

Since it was possible that the observed rhythmical fluctuations in the rate of CO_2 output were the result of a rhythm in the opening and closing of the stomata of the leaves, two experiments were carried out in which one or both epidermes were removed from the leaves.

Fig. 6*a* shows the effect of removing the lower epidermis from the leaves. The first peak of the rhythm occurred at 10 a.m., 18 hours after the commencement of darkness. The rhythm persisted with a phase and period closely similar to those of the rhythms in the control experiments carried out on whole leaves having an intact epidermis, although the amplitude of the first and second peaks was reduced.

Fig. 6*b* shows the result of removing both epidermes from the leaves and cutting the remaining mesophyll into pieces 1 cm.^{2} A persistent rhythm was present, closely similar in phase and period to those of both the previous experiment and the control experiment carried out on whole leaves.

The persistent rhythmical fluctuations in the rate of CO_2 output must therefore be a property of the leaf tissues in general and not merely the result of a rhythm in the size of the stomatal aperture.

Evidence for the endogenous nature of the rhythm and the effect of continuous illumination on the rhythm

The effect of continuous illumination on the rhythm was examined in a series of experiments in which pieces of mesophyll 1 cm.², stripped of their epidermes, were illuminated at an intensity of 3,000 lux for durations of 11, 22, 33, and 44 hours immediately following the normal period of daylight to which the plants had been subjected prior to the commencement of the experiment at 4 p.m. The broken line in Figs. 7A and 7B shows the result of the previous experiment in which darkness commenced at 4 p.m.

Fig. 7A, graph (1) shows the result of illuminating the tissue for 11 hours from 4 p.m. until 3 a.m. when the lights were extinguished. A rhythm commenced in which the first peak occurred at 7.30 p.m., 16·5 hours after the end of the light treatment. The rhythm was out of phase with the broken-line curve and maintained a 22-hour period. Fig. 7A, graph (2) shows that 22 hours illumination results in a rhythm in phase with the broken-line curve, having a first peak at 5.30 a.m., 15·5 hours after the end of the treatment.

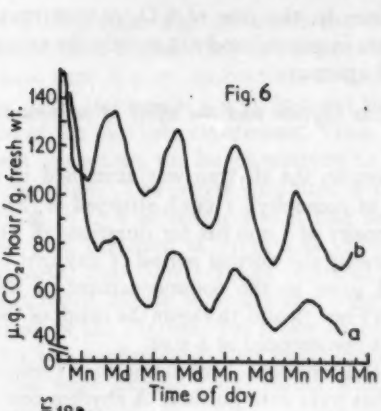
Closely similar results were obtained by illuminating the material for 33 and 44 hours. In the former case, Fig. 7B, graph (1), the first peak occurred 14·5 hours after the end of the treatment; the rhythm was out of phase with the broken-line curve. In the latter case, Fig. 7B, graph (2), the rhythm was in phase with the broken-line curve; the first peak occurred 14 hours after the end of the treatment and a second 23 hours later.

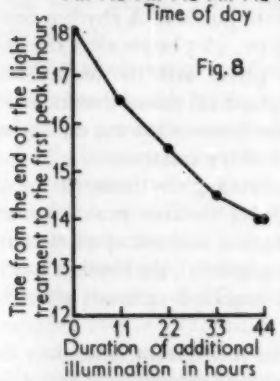
Light treatments at this intensity clearly inhibit the rhythm, but when the lights are extinguished the rhythm recommences; the first peak, and hence the phase of the rhythm, occurs a specific time after the end of the treatment, depending upon the duration of that treatment. The phase can thus be set at any desired time of day.

An interesting relation becomes apparent from these results. In the experiment in which the material received no additional illumination the time which elapsed between the commencement of darkness and the first peak (transient) was 18 hours. With additional light treatments of 11, 22, 33, and 44 hours the transient was reduced to 16·5, 15·5, 14·5, and 14 hours respectively. In general this relation, shown graphically in Fig. 8, indicates that the transient decreases as the length of the light treatment is increased.

DISCUSSION

The results presented in this paper can be divided into two groups, firstly those emerging from an investigation of the rate of CO_2 output of excised leaves of succulent plants in darkness and at a constant temperature and, secondly, those of preliminary experiments carried out upon an endogenous rhythm in the rate of CO_2 output of excised leaves of *Bryophyllum fedtschenkoi*.





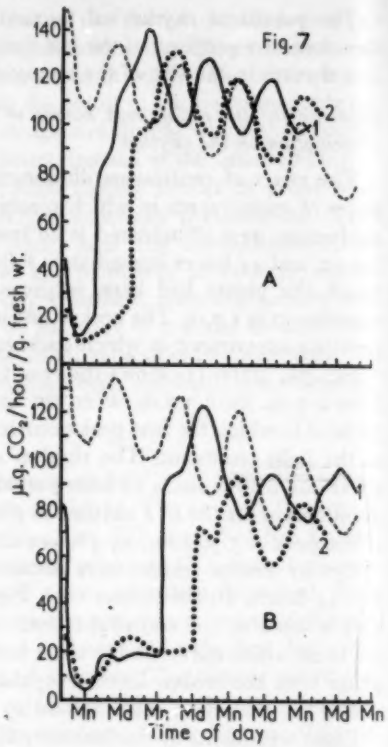


FIG. 6. (*a*) Shows the rhythm in leaves of *B. fedtschenkoi* in continuous darkness commencing at 4 p.m., at 26° C. and in an initially CO_2-free atmosphere when the lower epidermis has been removed. (*b*) Shows the rhythm in pieces of mesophyll 1 cm.², stripped of their epidermes, under identical conditions.

FIG. 7. Shows the effect of continuous illumination (3,000 lux) on the rhythm exhibited by pieces of mesophyll 1 cm.² which were stripped of their epidermes.

Broken line curve . . .	No additional light treatment, darkness commenced at 4 p.m.
A (1)	11 hours illumination from 4 p.m. to 3 a.m.
A (2)	22 ,, ,, ,, 4 p.m. to 2 p.m.
B (1)	33 ,, ,, ,, 4 p.m. to 1 a.m.
B (2)	44 ,, ,, ,, 4 p.m. to noon.

FIG. 8. Shows the decrease in the transient as the duration of the additional light treatment is increased.

The occurrence of persistent rhythms in the rate of CO_2 output of succulent plants is by no means a general phenomenon. Rhythms were observed in three species of *Bryophyllum* and in one species of *Sedum*. The infrequent occurrence of rhythms has been observed in other researches, Nuernbergk (1955), Ball and Dyke (1954), Ball, Dyke, and Wilkins (1957), Engel and Friederichsen

(1951, 1952, 1954), Pirson and Döring (1952), Ingold and Cox (1955), Ingold and Dring (1957), and Brandt (1953).

That the distribution of rhythms throughout the plant kingdom should appear to be so greatly restricted may denote either that the apparently sparse distribution is real or that rhythms are widespread, but present methods of detection are insufficiently sensitive. The present results, coupled with those of the papers noted above, clearly favour the first alternative.

In arhythmic species the graph representing the rate of CO_2 output with time follows a characteristic pattern. They exhibited one clearly defined peak which was followed by a gradual decrease in the rate of CO_2 output during the remainder of the experiment.

The observation of a persistent endogenous rhythm in the excised leaves of *B. fedtschenkoi* has not, to the author's knowledge, been previously described. The older definition of the term 'endogenous rhythm' as 'originating from within' has been placed on a more scientific basis by Pittendrigh (1954) who suggested that the following evidence was required to establish the endogenous nature of a rhythm:

(1) The persistence of the rhythm under constant conditions.

(2) The inversion and resetting of the phase of the rhythm by suitable treatments.

(3) That there should be some variation in the period from 24 hours even though this variation be slight.

The results presented in this paper clearly establish the endogenous nature of the rhythm in *B. fedtschenkoi*.

In common with rhythms described in other organisms the rhythm in *B. fedtschenkoi* possesses an almost constant period at 26° C. Pretreating the material with various alternations of light and darkness in 24-hour cycles had no effect upon the period of the rhythm in subsequent darkness; the phase was set by the time at which the lights were extinguished. The period was unaffected by the duration for which the rhythm was inhibited by continuous light.

The rapid disappearance of the rhythm in a stream of normal air suggests that the rhythm in the rate of CO_2 output may be due to a rhythm in the activity of a CO_2 absorption mechanism acting upon an approximately constant rate of CO_2 output. In a stream of normal air the material absorbed a considerable quantity of CO_2 on the first occasion (9 p.m. until 4 a.m.) that the acceptor system became active and, due to the presence of excess CO_2, became saturated. Except for one minor variation in the rate of CO_2 output no further uptake of the gas occurred. In a CO_2-free air-stream the acceptor mechanism became active at a similar time but, owing to the absence of excess CO_2, did not become saturated. The acceptor system then made repeated attempts at 22-hour intervals to satisfy its requirements.

It is worth while considering the possibility of a CO_2 acceptor mechanism actually being the time-keeping mechanism or 'master clock'. If this were the

case the 'clock' would be inhibited by CO_2. The scanty evidence in this paper for the CO_2 acceptor mechanism actually being the 'clock' is supplemented by the work of Schmitz (1951) who observed a weakly developed rhythm in the rate of CO_2 output of leaves of *Kalanchoë blossfeldiana* in a CO_2-free atmosphere and of Gregory *et al.* (1954) who did not detect a rhythm in this species in a stream of normal air. The persistence of a clearly defined rhythm in the petal movement of *K. blossfeldiana* in normal air (Bünsow, 1953) does not support this hypothesis. Clearly if the CO_2 acceptor mechanism is the 'master clock' then all external manifestation of rhythms must be inhibited in normal air. If, however, the CO_2 acceptor mechanism is not the 'clock' the effect of excess CO_2 would be to fully saturate the system which, although receiving periodic stimuli from the 'clock', would not become active again at 22-hour intervals. The 'clock' would function normally but its activity would not be manifest in the CO_2 metabolism of the leaves.

The discussion of the effect of light upon the phase of the rhythm is facilitated by considering a simple model system representing the basic time-keeping mechanism or 'clock'. In a simple system oscillating between two limits *A* and *B* the result of a light treatment would fall into one of the following categories: (1) Light may inhibit oscillation by driving the system to limit *A*. Darkness would allow the oscillations to recommence and the first post-treatment peak would occur a specific time after the end of the treatment. (2) Light may inhibit oscillation by causing the system to remain in the position it had reached when the stimulus was applied. In this case, at its simplest, a phase delay equal to the duration of the treatment would result. (3) Light may cause the rhythmical processes which are observed to be uncoupled from the 'clock'. Darkness would recouple the observed process to the 'clock' and the rhythm would retain the original phase.

Continuous illumination at an intensity of 3,000 lux completely inhibits the rhythm, but when the lights are extinguished the rhythm recommences, the first peak occurring a specific time after the commencement of darkness, depending upon the duration of the treatment. Clearly, it is the end of the light treatment that reinitiates the rhythm and sets the phase, indicating that light is inhibiting the endogenous 'clock' by driving it to one of its limits as outlined in case (1) above.

In common with the rhythms in *Avena sativa* (Ball and Dyke, 1954), *Kalanchoë blossfeldiana* (Schmitz, 1951), and *Gonyaulax polyedra* (Hastings and Sweeney, 1957) the end of the light treatment sets the phase of the rhythm in *Bryophyllum*. In several organisms, however, the commencement of the light period is the critical change that determines the phase of the rhythm (Flügel, 1949; Leinweber, 1956; Bünning, 1935; Pittendrigh, 1954; Engel and Friederichsen, 1951 and 1952; and Nuernbergk, 1955).

The gradual reduction in the transient as the duration of the light treatment is increased is a phenomenon which has not been generally observed. Armin, Prinz zur Lippe (1956), studying a rhythm in the CO_2 output of primary leaves of *Phaseolus multiflorus*, found that the longer the light period, in alternations

of light and darkness, immediately preceding continuous darkness the shorter the time from the commencement of darkness to the first peak. A close inspection of the results of Ball and Dyke (1954) reveal a similar, though less marked, relation between the duration of exposure of *Avena sativa* seedlings to red-light and the time from the commencement of darkness to the first peak of the rhythm in the growth-rate of the coleoptile.

These relations could be explained in terms of the simple oscillating system if, during inhibition resulting from prolonged illumination, there was a very gradual movement of the system from limit *A* to limit *B*. This would increase slowly with time and on extinguishing the light the system would recommence oscillations in the normal way by moving towards *B*. After prolonged illumination, however, the 'leak back' would result in the system having less far to travel to *B* than if it were actually at *A* when the lights were extinguished, and the first peak would therefore occur earlier.

The model oscillating system is introduced into this discussion merely to facilitate the interpretation and description of the effect of light upon the basic time-keeping mechanism present in the tissue. It is not intended to represent any specific chemical or physical process. Speculation on the precise nature of the endogenous time-keeping mechanism is inappropriate until more facts concerning its properties have been established. Further experiments towards this end are being conducted.

The author wishes to express his thanks to Sir Nigel Ball, Bt., under whose supervision this work was conducted and to Professor T. A. Bennet-Clark, F.R.S., for providing the facilities for the research and for his continued interest in its progress. I am indebted to the Royal Society for providing the funds for the purchase of the infra-red gas analyser and to the Department of Scientific and Industrial Research for a Research Studentship.

LITERATURE CITED

ARMIN, PRINZ ZUR LIPPE (1956). Über den Einfluss des vorangegangenen Licht-Dunkelwechsels auf die CO_2-Ausscheidung der Primärblätter von *Phaseolus multiflorus* in anschliessender Dunkelheit. *Z. Bot.* **44**, 297.

BALL, N.G., and DYKE, I. J. (1954). An endogenous 24-hour rhythm in the growth rate of the *Avena* coleoptile. *J. Exp. Bot.* **5**, 421.

—— DYKE, I. J., and WILKINS, M. B. (1957). The occurrence of endogenous rhythms in the coleoptiles in various cereal genera. Ibid. **8**, 339.

BRANDT, W. H. (1953). Zonation in a prolineless strain of *Neurospora*. *Mycologia*, **45**, 194.

BÜNNING, E. (1935). Zur Kenntnis der endonomen Tagesrhythmik bei Insekten und in Pflanzen. *Ber. d. bot. Ges.* **53**, 594.

BÜNSOW, R. (1953). Endogene Tagesrhythmik und Photoperiodismus bei *Kalanchoë blossfeldiana*. *Planta*. **42**, 220.

ENGEL, H., and FRIEDERICHSEN, I. (1951). Das Licht als Ursache periodische Guttationsschwankungen etiolierten Haferkeimlinge. Ibid. **39**, 309.

—— —— (1952). Weitere Untersuchungen über periodische Guttation etiolierten Haferkeimlinge. Ibid. **40**, 529.

—— —— (1954). Periodische Guttation bei *Zea mays*. Ibid. **44**, 459.

FLÜGEL, A. (1949). Die Gesetzmässigkeiten der endogonen Tagesrhythmik. Ibid. **37**, 337.

GREGORY, F. G., SPEAR, I., and THIMANN, K. V. (1954). The interrelation between CO, metabolism and photoperiodism in *Kalanchoë*. *Plant Physiol.* **13**, 669.

HASTINGS, J. W., and SWEENEY, B. M. (1957). The Gonyaulax Clock. In the *International Symposium on photoperiodism in plants and animals*, Gatlinburg, Tennessee. October 1957.

INGOLD, C. T., and COX, V. J. (1955). Periodicity of spore discharge in *Daldinia*. *Ann. Bot.* **19**, 201.

—— and DRING, V. J. (1957). An analysis of spore discharge in *Sordaria*. Ibid. **21**, 465.

LEINWEBER, F. J. (1956). Über die Temperaturabhängigkeit der Periodenlänge bei der endogenen Tagesrhythmik von *Phaseolus*. *Z. Bot.* **44**, 337.

NEEB, O. (1952). *Hydrodictyon* als Objekt einer vergleichenden Untersuchung physiologischer Grossen. *Flora*. **139**, 39.

NUERNBERGK, E. L. (1955). Über den zeitlichen Verlauf der Photosynthese bei Gewächshauspflanzen. *Die Gartenbauwissenschaft*. **1**, 391

PIRSON, A., and DÖRING, H. (1952). Induzierte Wachstumperioden bei Grünalgen. *Flora*. **139**, 314.

—— SCHÖN, J., and DÖRING, H. (1954). Wachstum- und Stoffwechselperiodik bei *Hydrodictyon*. *Z. Naturforsch*. **9b**, 349.

PITTENDRIGH, C. S. (1954). On the temperature independence in the clock system controlling emergence time in *Drosophila*. *Proc. Nat. Acad. Sci. U.S.A.* **40**, 1018.

SCHMITZ, J. (1951). Über Beziehungen zwischen Blütenbildung in verschiedenen Licht-Dunkelkombinationen und Atmungsrhythmik bei wechselnden photoperiodischen Bedingungen. *Planta*. **39**, 271.

SCHÖN, J. (1955). Periodische Schwankungen der Photosynthese und Atmung bei *Hydrodictyon*. *Flora*. **142**, 347.

SPOEHR, H. A., and MCGEE, J. M. (1923). *Studies in plant respiration and photosynthesis*. Carnegie Institute of Washington.

WILKINS, M. B. (1958). *Rhythmic phenomena in plants*. Ph.D. thesis, University of London, 1958.

Changes in the Chromosome Complements of Cells of *Vicia faba* Roots following Irradiation

DOUGLAS DAVIDSON

Department of Botany, University of Oxford

Received 28 November 1958

SUMMARY

1. Primary roots of *Vicia faba* were irradiated after one day's germination. The X-ray dose was 620 r.

2. Cells with changed chromosome complements appeared in lateral roots. It is inferred that these cells are descended from cells damaged by irradiation; chromosome breakage was seen in the first mitoses in the irradiated roots but was not scored.

3. Six types of chromosomal rearrangements were found. One type had been reported previously in primary roots of *V. faba* after X-raying.

4. The new complements appear to be stable because they occur in several cells of a lateral. Affected laterals could contain up to five new complements. The primordium of such a lateral must have consisted of five changed and at least one normal cell. It had previously been suggested that a root of *Vicia* had three or four initial cells.

5. The survival of the cells with changed complements is not due solely to successful competition with, nor to support by, normal cells, otherwise other types of changes should also have been found.

6. It is suggested that the changes found were perpetuated because they were induced in initial cells of the primary root, and that cells in which they were present became part of the primordia and continued as initial cells in apical meristems of lateral roots.

7. Apical cells appear to have a lower sensitivity than other meristematic cells, so providing, after treatment, a reservoir of normal cells from which regeneration can occur.

INTRODUCTION

AFTER irradiation or treatment with certain chemicals breaks may be seen in the chromosomes of dividing cells. The percentage frequency of cells with chromosome breaks reaches a maximum, in *Vicia faba* for example, within 1 to 2 days after treatment, and then decreases; there is at the same time an increase in the frequency of normal or apparently normal cells in division (Thoday, 1951). The normal cells may include cells which have undergone chromosome changes but in which the changes are not visibly detectable, e.g. reciprocal exchanges of portions of chromosome arms of exactly equal lengths.

Cells with many fragments may complete only one division after treatment. The loss of fragments at anaphase leads to cell death or to failure to divide again. Other damaged cells do divide, but less and less frequently. Perhaps the change in chromosome structure that they underwent has been accompanied by a reduction in metabolic efficiency and in competing with normal cells for substrates they are at a disadvantage; because they are less efficient they may

divide less and less frequently. Alternatively, changed chromosomes will not be seen when the affected cells enter the zone of differentiating and non-dividing cells.

Though in general cells with changed chromosome complements do not continue to appear in division in actively growing roots they do occasionally survive for several days or even weeks. In *Allium*, dicentric chromosomes were seen at anaphase eight days after X-raying (Sax, 1941). In *Vicia faba* and *Crepis capillaris* roots, changes which appear to be stable have been found; many cells of each affected root showed the same change when examined 3 weeks after irradiation (Brumfield, 1943; Richard, 1952). Few new complements survived, however, and they were all of a similar type. It remained to be seen whether other stable changes could be induced in *Vicia*; and also whether the altered complements could occur in lateral roots if the irradiation had been delivered to the primary root many days and many cell generations previously.

MATERIAL AND METHODS

Beans (*Vicia faba*, variety Carter's Aquadulce Longpod) were germinated in distilled water at 25° C. After 1 day beans whose radicals had emerged were irradiated. They were planted in sterilized sand and kept at 25° C. After 4 days they were washed and suspended over aerated distilled water in a temperature cabinet at 20° C.

Some primary roots were fixed when the first mitoses were taking place, but mainly lateral roots about 1 cm. long were used. Roots were pretreated in 0·01 per cent. colchicine for 3 hours and then fixed in 2BD or acetic alcohol (1/3). They were prepared as Feulgen squashes after acid hydrolysis. No sections were used. First, because chromosome morphology is more accurately determined in squash preparations. Secondly, because it would be difficult or impossible to distinguish between genuine chromosome deficiencies and cut chromosomes.

The X-ray dose was 620 r delivered in 5 minutes at 25 cm. from a Newton–Victor machine operating at 5 ma. and 70 Kvp. The survival rate of beans after this dose of X-rays was low. Lateral roots developed on only 3 out of 70 beans. The time for lateral roots to develop was extended from the normal time of about 10–12 days to 3–4 weeks after irradiation. The high death-rate of roots in these experiments agrees with the observation that the LD50 dose for primary roots of *Vicia* is 435±15 r (Gray, Read, and Poynter, 1943).

The 12 chromosomes of *Vicia faba* have been figured (McLeish, 1953) and described (Davidson, 1957) before; the same terminology will be used here.

BROKEN CHROMOSOMES IN PRIMARY ROOTS

The first cells to enter division after irradiation showed chromosome breakage and reunion. No quantitative data of the numbers of breaks was collected, only the general pattern of breakage was noted. Centric and acentric fragments of all the lengths that could be derived from the chromosomes of *Vicia* were present. From centric fragments it could be shown that breaks had occurred

in S and M chromosomes, and from fragments which included the nucleolar constriction it could be shown that the nucleolar arm of the M chromosome had been broken. Breakage had occurred in over half of the cells seen in division. In the cells with breaks there was an enormous variation in the structure of the chromosome complement. Most of this variation was due to the distribution of the breaks, but part of it was due to the reunions which followed breakage. The translocation of chromosome segments had given rise to monocentric and to dicentric chromosomes that do not occur in normal cells of *Vicia*. Dicentric chromosomes derived from S:S reunions, S:M reunions, or M:M reunions were all seen. Some of the new chromosomes that are found in *Vicia* in X1 and X2 divisions following irradiations have been shown (Revell, 1955; Davidson, 1958, Fig. 3).

Finally, it was noted in some non-dividing cells that micronuclei were present. A micronucleus may be formed by an acentric fragment which does not move to a pole at anaphase and so is not included in either of the 2 nuclei reconstituted at telophase. Cells containing deficient nuclei do not usually divide often after the loss of the chromosome segment.

The usual gradual reduction in the number of abnormal cells in division and the successive changes, if any, of the breakage patterns seen in dividing cells in the primary root were not followed on this occasion. The ability of cells with new non-lethal complements to maintain themselves was judged solely from their presence in lateral roots.

NEW CHROMOSOMES IN LATERAL ROOTS

New complements appeared, along with normal complements, in dividing cells in lateral roots. This confirms Brumfield's finding that cells with altered complements can survive over many cell generations. It also shows that cells with new complements can become part of the meristems from which the lateral roots develop.

In all, six types of changed chromosome complements were found. They have been numbered for convenience (Fig. 1). Type V is the only exchange which is certainly between non-homologous chromosomes. Types III and IV may involve homologous chromosomes. Two type IV changes involving homologous chromosomes were the only changes found by Richard (1952). All of the new complements are alike in certain respects:

1. Never more than two of the original 12 chromosomes have been involved in each rearrangement.

2. No matter what the form of the change, the original number of centromeres has been strictly maintained.

3. The total length of the chromosome complement has not been markedly reduced; even in cells with deficiencies, only small segments have been lost.

4. Homozygous deficiencies were not found.

5. No cells were seen that were heterozygous for a deficiency which included the nucleolar constriction.

6. No dicentric chromosomes were found.

Unequal translocations which would produce chromosomes with arms longer than half the length of the normal cell were not found. Such translocations would result in incomplete separation of chromatids and bridge formation at anaphase. Cells affected in this way would probably stop dividing. The absence of cells with chromosomes with unusually long arms confirms the view that cell or spindle length and arm length are mutually adjusted.

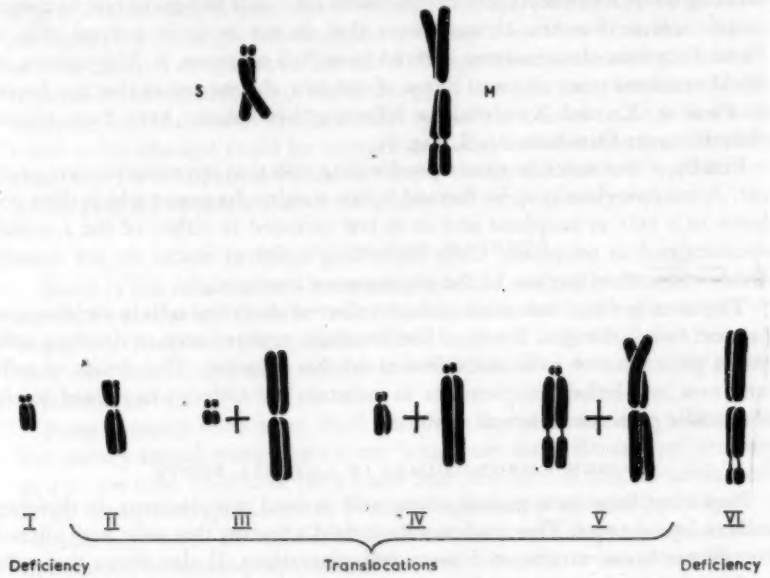


FIG. 1. The two types of normal chromosomes (S and M) and the six types of rearrangement found in *Vicia* roots after X-raying. ×1,600.

As might have been predicted from our knowledge of the centromere as an essential component of a chromosome which completes a successful anaphase, no acentric fragments were found to have perpetuated themselves. Of the many types of centric fragments only two have survived: one has a deficiency for almost half a long arm of an S chromosome (I) and the second a deficiency for a small terminal segment of the nucleolar arm of an M chromosome (VI). The absence of all but a few changed monocentric chromosomes and of all the dicentric chromosomes indicates that most of the cells with new chromosomes which were found immediately after X-raying either did not continue to divide, or did not become part of the meristems from which the lateral roots were derived.

New complements were never frequent in the lateral roots (Table I). In all, 20 per cent. of the cells in affected laterals had undergone obvious changes. In different laterals of one bean and from laterals of different beans, the same six types of chromosome changes occurred repeatedly. Furthermore, the only

type of change found by Brumfield (1943), an unequal translocation between a long arm and a short arm of two S chromosomes, appeared in affected laterals from all three beans which grew in the present experiments (Fig. 1, Type III; Table I).

Micronuclei were not seen in the cells of the lateral roots (cf. cells of primary roots seen after irradiation where micronuclei were present). Evidently all secondary changes which may appear at X2 and X3 mitosis had occurred by

TABLE I

The frequency of cells with normal and changed chromosome complements in lateral roots of Vicia *after X-raying the primary root*

Bean and root no.	Normal cells	Type of chromosome change						Total abnormal cells	Total no. cells
		I	II	III	IV	V	VI		
A1	63	—	8	—	—	—	—	8	71
A2	75	1	—	6	—	—	—	7	82
A3	52	—	8	—	—	—	2	10	62
A4	NORMAL								
B1	45	4	—	2	1	—	—	7	52
B2	40	3	11	10	—	10	3	37	77
B3	23	—	—	—	3	—	—	3	26
C1	26	—	4	3	1	4	—	12	38
C2	NORMAL								
C3	NORMAL								
Total	324	8	31	21	5	14	5	84	408

the time of fixation of the lateral roots and the complements had become stable. The conclusion that the new complements are stable also follows upon the presence of each complement in several cells of the same root (Table I, Pl. II, A and B). The new complements may be considered to have been formed in the primary root and to have been in constituent cells of the lateral roots during later development. They are also complements which have undergone a number of cell divisions since the laterals were about 1 cm. in length at the time of fixation. The presence in one root (Table I, root B2) of five different complements, together with normal cells, shows that at least 6 cells formed the primordium from which this lateral root was formed. Perhaps, as there are more than 50 per cent. of normal cells in the root, there were several normal cells in the original meristem and more than 6 initial cells. In other roots at least 4 or 5 cells formed the root primordium. Brumfield (1943) estimated from a study of primary roots which were similar chromosome chimeras that there were only 3 or 4 cell initials in the meristem. This estimate was based on the distribution of a single type of chromosome change, which occurred in 4 of the 27 roots X-rayed. Results from primary roots of *Vicia* (Davidson, unpublished) show that as many as 11 types of new complements can occur 9 and 11 days after irradiation, and therefore there must have been more than 3 or 4 initial cells. The new complements sometimes included dicentric chromosomes.

DISCUSSION

The survival of new complements. A root tip is a complex structure in which the cell is the basic unit of structure and the visible unit of growth. The cells of a root are not all alike; they differ in their chemical composition (Jensen, 1956), enzyme content (Robinson and Brown, 1952), rates of DNA synthesis (Clowes, 1956), and in rates of division (Gray and Scholes, 1951). If the cells with chromosome deficiencies are supported, and receive metabolites from the surrounding normal cells, then several of the various types of deficient chromosomes seen in the primary roots might have been expected to survive. The cells with dicentric chromosomes which also have deficiencies due to the original losses and which may become further unbalanced from bridge–breakage–fusion cycles might also have been expected to survive. Yet dicentric chromosomes, though they would have been immediately obvious, were not found again after their occurrence in the primary roots. Only two types of deficiencies were found (Types I and VI), both were in the heterozygous condition. A heterozygous deletion does not always prevent cell multiplication, a whole clone of *Hyacinthus* occurs in this condition (var. Madame du Barry, Darlington *et al.*, 1951). Thus the absence of dicentric chromosomes and the presence of only two types of deficient chromosomes make it unlikely that the damaged cells survive because they are supported by normal cells; it seems that other factors affect the survival of cells with new complements.

The other changes (Types II–V) would all be expected to survive provided there was no genically determined lethality due to a position effect following translocation. A bean in which all the cells had the type V change has been described (Ockey, 1957). Similar changes are known from other plants and animals. Since all the types of new chromosomes which have maintained themselves over many cell generations in the treated *Vicia* roots occur in all the cells of some adult individuals their survival in the roots may be due to their ability to compete successfully with normal cells and unsuccessful competition with normal cells may be the cause of the failure of other changed complements to survive. However, though competition may explain the failure of some cells to survive, it does not explain why so few changes survived, out of the large number of similar types of change (i.e. involving translocation or loss) in the primary root.

The survival of certain, mainly normal cells, in roots of *Luzula* after heavy doses of X-ray (20,000–160,000 r) has been attributed to the selection of the

---

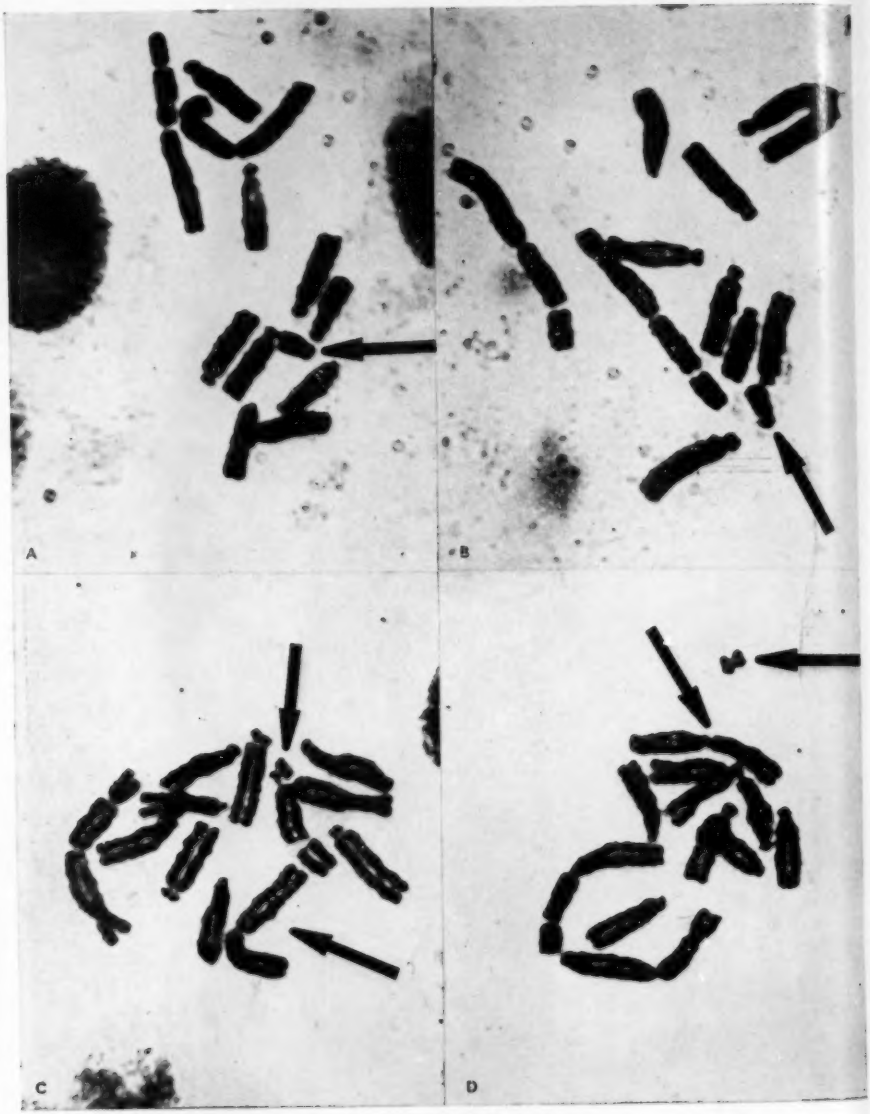
PLATE I

New S chromosomes (with arrows). (A), (B), (C), with centromeres that are now sub-median, rather than sub-terminal in position. (D) with a secondary constriction. This is the result of an exchange between an S and an M chromosome. × 1,300 (approx.).

PLATE II

(A) and (B) are two cells from one lateral root. Both contain 2M, 9S and 1 deficient S chromosome (with arrow). (C) and (D) are taken from laterals of different beans. Both contain a new M and a new very short chromosome (with arrows). The new M, unlike the normal M chromosomes, has no secondary constriction. × 1,300 (approx.).

D. DAVIDSON—PLATE I



D. DAVIDSON—PLATE II

least injured cells (Nordenskiold, 1957), but the concept of intra-somatic selection (Ehrenberg *et al.*, 1953; Nybom *et al.*, 1953) was originally related to cells with gene mutations, and it was thought that 'chromosome aberrations and rearrangements are not responsible for the chief part of the elimination' (i.e. of the affected cells). In *L. capitata* the number of abnormal metaphases per root decreased slightly with time after X-raying, indicating that normal cells may have been at an advantage. But there was no corresponding decrease in roots of *L. pallescens*; the phenomenon is therefore not one of general occurrence.

In discussing the factors that affect the survival of the new complements it was assumed that the dividing cells in which chromosome changes were seen soon after irradiation are the cells from which subsequent growth occurred. It was inferred, in comparing the breakage pattern seen in the primary and lateral roots, that all the original chromosome changes would survive if they were able to, as though all of the cells from the apex constituted the meristem from which growth occurs. This is not so, however (Clowes, 1956). The region of general meristematic activity towards the apex of the root gives rise to cells which have a limited period of growth and division; they will eventually enter the zone of differentiating cells. It is only the cells at the very apex of the root, i.e. the initials, which continue to divide, and they give rise to cells which enter the region of general meristematic activity. Thus if a new chromosome is to survive and appear in the meristem at successive times it seems that it must be present in an initial cell of the root. It is suggested therefore that the new chromosome complements found in lateral roots are not, of the many changed complements found in the primary roots, the only ones which were able to survive a period of selection; they are non-lethal changes which were induced in the initial cells of the apices of the irradiated roots. Later the cells with changed complements were included in the initial cells of the developing lateral roots. Brumfield (1943) also considered that the new complement he observed in *Vicia* had been induced in cells of the apex, but he concluded that there were 3 or 4 central cells, rather than a number of initial cells and a quiescent centre (Clowes, 1956).

The frequency of abnormal cells seen in division in meristems after irradiation gradually decreases. If, as seems likely, the source of the normal cells is the cells at the apex, it suggests that these cells are less sensitive to irradiation than the cells of the general meristem. It is known that the cells at the apex do not divide often (Clowes, 1956) and this may be partly responsible for their low sensitivity. Resting stage cells of *Vicia* are less sensitive to irradiation than cells actually in division (Davidson, 1958). Furthermore, the long resting stage of apical cells will facilitate the processes of restitution and repair.

Quite apart from the different sensitivities of the cells at different stages of the mitotic cycle, there may be other factors which make the apical cells more resistant to treatment than other cells. Such a differential sensitivity within a short apex would probably be advantageous. Recovery could occur after treatment because of the reservoir of normal or less damaged cells.

The gradual increase in the number of normal cells that occurs after X-raying also occurs after treatment with colchicine or a division inhibitor, phenyl mercuric acetate (unpublished results). After treatment with colchicine, tetraploid cells and multi-nucleate cells appear but are gradually replaced by diploid cells. It was found that regeneration of the normal diploid tissue occurs from the root apex (Levan, 1938), from cells which must have been unaffected by the treatment.

It is suggested that the apical cells of the root of *Vicia* are less sensitive to experimental treatments than the cells of the general meristem. These cells confer upon the root a buffering or homeostatic ability. But when chromosome changes are induced in the apical cells they are perpetuated, if non-lethal, and continue to appear in the general meristem.

I am grateful to Dr. F. A. L. Clowes for helpful discussions and to the Nuffield Foundation for financial assistance. The X-ray machine used in these experiments was purchased with the help of a Grant-in-Aid from the Royal Society.

LITERATURE CITED

BRUMFIELD, R. T. (1943). Cell linkage studies in root meristems by means of chromosome rearrangements induced by X-rays. *Amer. J. Bot.* **30**, 101.

CLOWES, F. A. L. (1956). Localization of nucleic acid synthesis in root meristems. *J. Expti. Bot.* **7**, 307.

DARLINGTON, C. D., HAIR, J. B., and HURCOMBE, R. (1951). The history of the garden hyacinths. *Heredity*, **5**, 233.

DAVIDSON, D. (1957). The irradiation of dividing cells. I. The effects on prophase chromosomes. *Chromosoma*, **9**, 39.

—— (1958). II. Changes in sensitivity to X-rays during mitosis. *Ann. Bot.* N.S. **22**, 183.

EHRENBERG, L., GUSTAFSSON, A., LINDQVIST, U., and NYBOM, N. (1953). Irradiation effects, seed soaking and oxygen pressure in barley. *Hereditas*, **39**, 493.

——, ——, and VON WETTSTEIN, D. (1956). Studies on the mutation process in plants—regularities and intentional control. In *Conference on Chromosomes*. Wageningen.

GRAY, L. H., READ, J., and POYNTER, M. (1943). The effect of ionizing radiations on the broad bean root. *Brit. J. Radiol.* N.S. **16**, 125.

——, and SCHOLES, M. E. (1951). The effect of ionizing radiation on the broad bean root. VIII. Growth rate studies and histological analyses. *Brit. J. Radiol.* N.S. **24**, 82, 176, 228, 285, and 348.

JENSEN, W. A. (1956). On the distribution of nucleic acids in root tips of *Vicia faba*. *Exptl. Cell Res.* **10**, 222.

LEVAN, A. (1938). The effect of colchicine on root mitoses in Allium. *Hereditas*, **24**, 471.

MCLEISH, J. (1953). The action of maleic hydrazide on *Vicia*. *Heredity* (Suppl.), **6**, 125.

NORDENSKIOLD, H. (1957). Changes in somatic chromosome patterns in root tips of X-ray treated *Luzula*. *Kungl. Lantbrs. Annaler*, **23**, 273.

NYBOM, N., LINDQVIST, V., GUSTAFSSON, A., and EHRENBERG, L. (1953). Biological effects of X radiation at low temperatures. *Hereditas*, **39**, 445.

OCKEY, C. H. (1957). An interchange heterozygote in *Vicia faba*. *Nature*, **179**, 740.

REVELL, S. H. (1955). A new hypothesis for chromatid changes. *In Radiology Symposium*. Ed. F. Bacq and P. Alexander. Butterworth, London, 1955, 243.

RICHARD, W. H. (1952). *X-ray induced chromosomal aberrations and root histogenesis in Crepis capillaris* (L.) Wallr. M.A. Thesis. University of Colorado.

ROBINSON, E., and BROWN, R. (1952). The development of the enzyme complement in growing root cells. *J. Exptl. Bot.* **3**, 356.

SAX, K. (1941). An analysis of X-ray induced chromosomal aberrations in *Allium* root tip cells. *Genetics*, **26**, 418.

THODAY, J. M. (1951). The effect of ionizing radiations on the broad bean root. *Brit. J. Radiol.* **24**, 572 and 622.

Cultivation of Excised Anthers *in vitro*—Effect of Nucleic Acids

I. K. VASIL

Department of Botany, University of Delhi, Delhi 8, India

Received 15 September 1958

SUMMARY

Anthers of *Allium cepa* and *Rhoeo discolor*, excised at leptotene-zygotene and diplotene-diakinesis, have been cultured in modified White's medium supplemented with different concentrations of ribonucleic acid and desoxyribonucleic acid. The best development was obtained with ribonucleic acid in which there was a 100 per cent. survival of the microspore mother cells up to the formation of 1-celled microspores. In *R. discolor*, the time taken by the microspore mother cells to form tetrads *in vitro* was half of that required in nature. All attempts to induce division of the microspore nucleus *in vitro* failed.

INTRODUCTION

DURING the past decade some attempts have been made to culture excised anthers *in vitro* and a variety of media has been tried for this purpose. However, in most cases anthers excised at the pachytene or diplotene-diakinesis stage fail to develop beyond the 1-celled microspore stage and attempts to culture pre-leptotene anthers have been unsuccessful. So far, the best development of anthers has been obtained in *Trillium erectum* with the help of 25–50 per cent. coconut milk (Sparrow *et al.*, 1955; *see also* Walker, 1957). The beneficial effect of coconut milk on anther survival is believed to be due to its nucleic acid content (Linskens, 1956*a*).

A correlation between the development of the microspore mother cells and the synthesis of nucleic acids in the anthers is indicated by the work of several investigators. Sparrow *et al.* (1952) showed an increase in the DNA content between pachytene and diplotene; after this there was no increase during the first or the second divisions. From this it may be assumed that the premature death of anthers *in vitro* without coconut milk is due to the lack of DNA (Linskens, 1956*a*). In most plants the meiotic synthesis of DNA is completed by diplotene so that anthers excised at or after diplotene give little difficulty in culture. Therefore, it seems that the most important steps in the differentiation of the microspore mother cells occur during the premeiotic interphase and in early leptotene. After this stage all that is required are the conditions for survival, except in those forms where growth occurs during pachytene-diplotene (e.g. *Tradescantia*).

In *Lilium longiflorum* DNA synthesis is completed even before leptotene (Taylor, 1953) and the failure of microspore mother cells to undergo further

development indicated that some other factor is limiting. Since in this plant the development of the tapetum is not complete until early diplotene this may be one of the factors limiting the development of pollen (*see* Taylor, 1950, 1953; Taylor and McMaster, 1954). A similar correlation between the development of the tapetum and the microspore mother cells has been shown in *Idesia polycarpa* (Linskens, 1956*a*).

All food materials to the sporogenous tissue must pass through the tapetum which consists of large, extremely active and richly cytoplasmic cells. Cooper (1952) reported that during meiosis in *Lilium henryi* and *L. regale*, DNA is transferred from the tapetal cells to the microspore mother cells. These observations could not be confirmed by the autoradiographic and microphotometric studies of Taylor and McMaster (1954) who attributed Cooper's results to be due to artifacts caused by fixation. More recently, however, Linskens (personal communication; *see also* Linskens, 1958) has found that in *L. henryi* after the beginning of prophase, DNA appears in the form of nucleotides, perhaps from the degenerating tapetal cells and nuclei, and is taken up by the already divided mother cells for synthetic processes.

The location, structure, and function of the tapetum make it a tissue of considerable physiological and morphological significance. Steil (1950) has shown that enzymes from the tapetal cells sometimes interrupt meiosis resulting in partial or complete degeneration of the microspore mother cells. This emphasizes the importance of the tapetum in the nutrition and development of pollen and Linskens (1956*a*) suggests that further biochemical work should be done on this problem (*see* Linskens, 1956*b*). One way of tackling it would be to culture squeezed-out microspore mother cells so that the effect of the tapetum and the wall layers is eliminated. Side by side with this, there has to be a detailed examination of the development of the tapetum and the nature of its chemical contents.

EXPERIMENTAL MATERIAL AND TECHNIQUE

Plants of *Allium cepa* and *Rhoeo discolor* were grown in the Delhi University Botanical Garden under normal field conditions prevailing at Delhi. Inflorescences were brought to the laboratory at 8–9 a.m. One anther[1] was removed from each bud and a quick iron-acetocarmine squash was prepared to check the stage of development. The buds were then sterilized in a clear 8 per cent. solution of calcium hypochlorite for 3 minutes and washed twice in sterile

[1] It was found that all the six anthers in a bud are approximately at the same stage of development (similar observations were made in *Lilium longiflorum* by Ogur *et al.*, 1951). It was also observed that while in anthers developing *in vivo* the meiotic divisions are mostly synchronous, in anthers cultured *in vitro* several stages of meiosis could be seen in a single anther.

Although there is a correlation between the length of the bud and the stage of development of its anthers, Taylor's method of estimating meiotic stages on the basis of bud length was found to be unreliable. For example, in *Allium cepa*, buds of a particular size which show microspore mother cells during the peak of the flowering season show 1-celled microspores towards the end of the flowering season. Even at a given time there are variations in the size of the buds containing anthers at a particular stage of development. Similar results have been obtained by Linskens (1956*b*) in *Lilium henryi*.

double distilled water. The anthers were then dissected out (under a stereo-microscope) and inoculated in Pyrex culture tubes (size 3 in.×1 in.; containing 10 ml. of the nutrient medium) under aseptic conditions.

All the media were prepared in Pyrex double distilled water and their pH was adjusted to 5·5 (with the help of 0·05 N. NaOH). Sterilization of the media was done in an autoclave at 15 lb. pressure for 20 minutes.

White's modified medium (WM) with the following composition was used. (i) *Major element solution* (in g./l.): magnesium sulphate, 3·6; calcium nitrate, 4 H_2O, 2·6; sodium sulphate, 2·0; potassium nitrate, 0·8; potassium chloride, 0·65; sodium dihydrogen phosphate, H_2O, 1·65. (ii) *Trace element solution* (in mg./l.): manganese sulphate, 4 H_2O, 3,000; zinc sulphate, 7 H_2O, 500; boric acid, 500; copper sulphate, 5 H_2O, 25; sodium molybdate, 2 H_2O, 25; cobalt chloride, 25; and 0·5 ml. conc. sulphuric acid (sp. gr. 1·83) in 1 litre. (iii) *Ferric citrate solution*, 2·5 g./l. (iv) *Vitamin and amino-acid solution* (mg./100 ml.): glycine, 150; niacin, 25; thiamin hydrochloride, 5; pyridoxine hydrochloride, 5; calcium pantothenate, 5. (v) *Indoleacetic acid*: 100 mg. in 100 ml. ethanol.

The final medium consisted of: Solution (i) 100 ml.; Solution (ii) 1 ml.; Solution (iii) 4 ml.; Solution (iv) 5 ml.; Solution (v) 2 ml.; Agar, 8 g., and Sucrose, 10 g., made up to 1 litre.

The cultures of *Allium cepa* were stored at 20° C. and those of *Rhoeo discolor* at 25° C. All the cultures were exposed to diffused laboratory light. At least two dozen cultures, each containing 5 anthers obtained from a single bud, were made for each of the tests. The tests were usually replicated after about a fortnight. Two anthers were removed aseptically from each culture tube at different intervals and examined with the help of iron-acetocarmine squashes.

EXPERIMENTAL RESULTS

1. *Allium cepa.* As shown in an earlier communication, the anthers of *A. cepa* normally do not show any development in White's medium (Vasil, 1957 *a*, *b*). In nature, buds having anthers at leptotene-zygotene (Fig. 1) form tetrads in 2–3 days.

(*a*) *White's medium supplemented with ribonucleic acid* (*RNA*).[1] Out of the four concentrations used (10, 25, 50, and 100 mg./l.), 50 mg./l. RNA proved to be the best for the development of anthers (Table I).

Anther's excised at leptotene-zygotene and cultured in WM with 50 mg./l. RNA showed 98 per cent. tetrads along with 2 per cent. degenerated cells after 4 days. In the same medium anthers excised at diplotene-diakinesis formed 100 per cent. tetrads after 4 days (Fig. 4; *see also* Figs. 2 and 3). This was by far the best development obtained *in vitro* with the anthers of *Allium cepa* excised at leptotene-zygotene or diplotene-diakinesis. In a medium containing 100 mg./l. RNA there was inhibition of pollen development.

[1] RNA (sodium ribonucleate) was used after dissolving in double distilled water with the help of 0·05 N. NaOH. It was used as received from the manufacturers (B.D.H.) and contained no desoxyribose or free ribose.

TABLE I

Development of anthers, excised at leptotene-zygotene (LZ) or diplotene-diakinesis (DD), of Allium cepa in White's medium with RNA and DNA additions

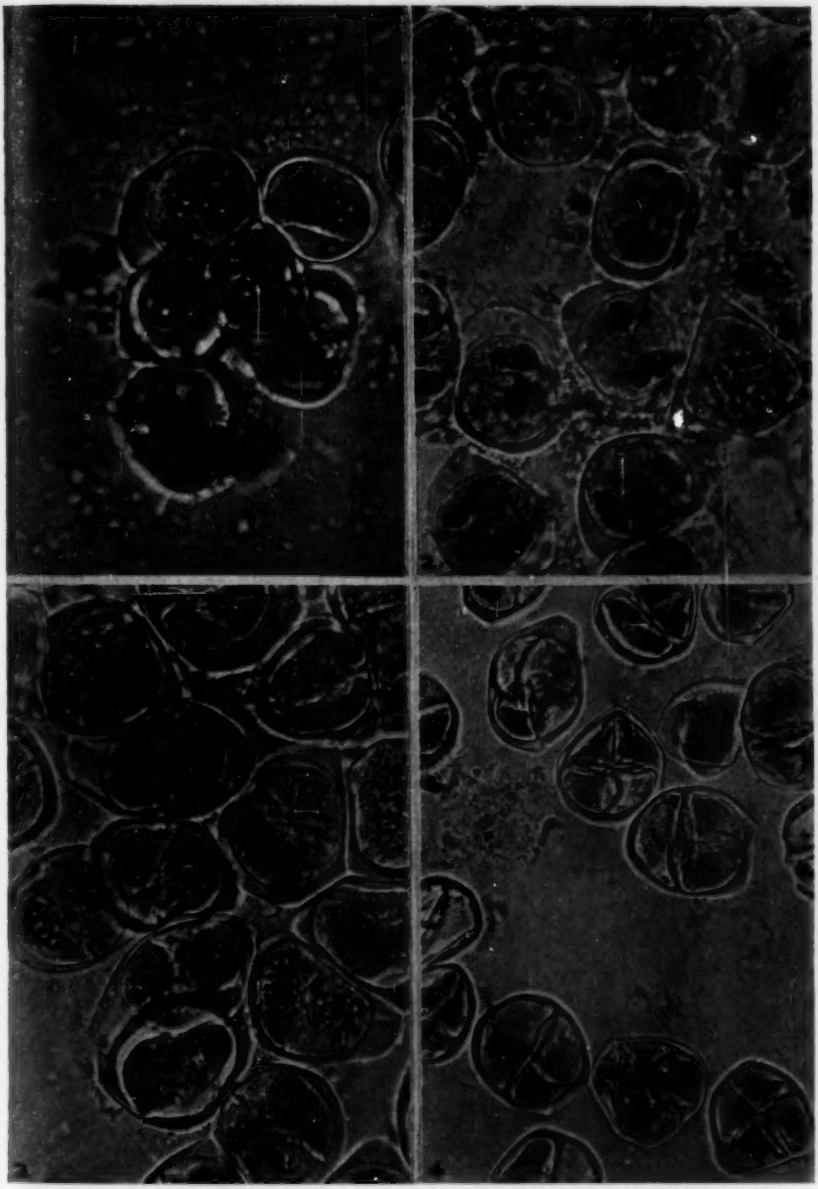
Figures are percentages found at the following stages: leptotene-zygotene (LZ), diplotene-diakinesis (DD), metaphase I (MI), dyads (Dy), Metaphase II (MII), 2-celled with 4 nuclei (2C), tetrads (Td).

RNA mg./l.	Days	Excised at	LZ	DD	MI	Dy	MII	2C	Td
10	1	LZ	60	34	—	6	—	—	—
		DD	—	26	—	12	33	7	22
25	1	LZ	—	72	6	16	—	6	—
		DD	—	15	—	38	—	6	41
	2	LZ	—	32	—	43	—	8	17
		DD	—	32	—	43	—	8	17
50	1	LZ	12	10	—	68	—	—	10
		DD	—	2	—	22	—	—	76
	2	LZ	—	—	—	16	—	—	84
		DD	—	—	—	6	5 (telo II)		89
	4	LZ	2	—	—	—	—	—	98
		DD	—	—	—	—	—	—	100
DNA									
10	1	LZ	—	32 (68 degenerated)			—	—	—
		DD	—	—	—	42 (58 degenerated)			—
25	1	LZ	38	62	—	—	—	—	—
		DD	—	50	—	32	13	—	5
	2	LZ	36	47	—	17	—	—	—
		DD	—	42	—	20	30	—	8
50	1	LZ	7	32	—	39	22 (telo II)		—
		DD	—	3	—	29	58	—	10
	2	LZ	5	24	—	46	—	—	25
		DD	—	3	—	21	56	—	20

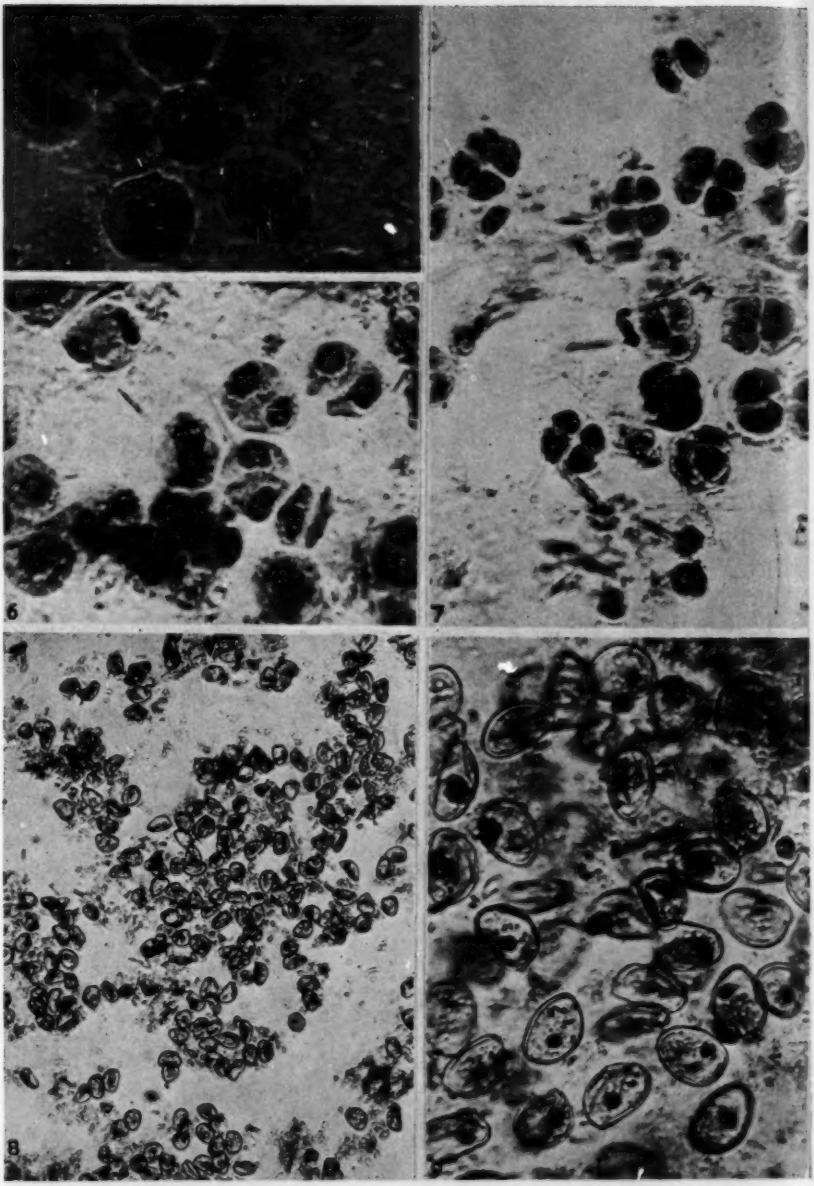
(*b*) *White's medium supplemented with desoxyribonucleic acid (DNA).*[1] In this medium also best development was obtained at a concentration of 50 mg./l. However, as shown in Table I, the survival of microspore mother cells was not as good in this medium as in the medium containing RNA. In White's medium with 100 mg./l. DNA no development was seen and degeneration of anthers occurred within two days.

2. *Rhoeo discolor*. In nature, anthers at leptotene-zygotene or diplotene-diakinesis (Fig. 5) take 24 hours to form dyads and 48 hours are required to produce young tetrads. Anthers excised at leptotene and cultured in White's medium showed 36 per cent. degenerating mother cells, 35 per cent. at diplotene-diakinesis and 29 per cent. dyads after 1 day. When excised at diplotene-diakinesis, they showed 33 per cent. degenerating mother cells, 24 per cent. mother cells at diplotene-diakinesis and 43 per cent. dyads after 1 day. In both the cases no further development occurred and the anthers gradually degenerated. During the degeneration of the anthers of *Rhoeo*

[1] DNA (Na salt; ex herring sperm) was used after dissolving in double distilled water with the help of 0·05 N. NaOH. The sample was used as received from the manufacturers (L. Light & Co., England). Chromatographic tests revealed no contamination with kinetin, a cell division factor, originally obtained from DNA.



FIGS. 1–4. *Allium cepa*. Fig. 1. Microspore mother cells at the stage of excision, leptotene-zygotene. ×460. Fig. 2. Excised at diplotene-diakinesis and cultured in WM with 10 mg./l. RNA; after 1 day. Dyads and mother cells. ×600. Fig. 3. Excised at leptotene-zygotene and cultured in WM with 50 mg./l. RNA; after 1 day. Prophase II. ×540. Fig. 4. Excised at diplotene-diakinesis and cultured in WM with 50 mg./l. RNA; after 4 days. All tetrads. ×430.



FIGS. 5–9. *Rhoeo discolor*. Fig. 5. Stage of excision; diplotene-diakinesis. ×710. Fig. 6. Excised at leptotene and cultured in WM with 25 mg./l. RNA; after 1 day. ×675. Fig. 7. Excised at diplotene-diakinesis and cultured in WM with 25 mg./l. RNA; after 1 day. Compare with Fig. 6. ×535. Fig. 8. Excised at diplotene-diakinesis and cultured in WM with 25 mg./l. RNA; after 2 days. 1-celled microspores. Compare with Fig. 7. ×148. Fig. 9. Excised at the 1-celled microspore stage and cultured in WM with 25 mg./l. RNA; after 1 day. The microspore nucleus has already started degenerating and the cytoplasm in the microspore is scanty. ×535.

discolor in vitro a light-brown pigment, which later becomes dark brown, appears in some of the cells near the region of the connective. Gradually this pigment spreads in other cells of the anther wall and then in the microspore mother cells.

(*c*) *White's medium supplemented with ribonucleic acid.* The optimum development of the anthers of *Rhoeo discolor*, excised at leptotene-zygotene or diplotene-diakinesis and cultured in White's medium with different concentrations of ribonucleic acid, is shown in Table II (*see also* Figs. 6–9). However, the best development of the anthers was seen with 25 mg./l. RNA where anthers excised at leptotene-zygotene showed 3 per cent. undeveloped mother cells, 76 per cent. dyads, and 21 per cent. tetrads after 2 days. In the same medium, anthers excised at diplotene-diakinesis showed 100 per cent. tetrads after 1 day and 1-celled microspores after 2 days. The time taken for the production of tetrads (when excised at diplotene-diakinesis) is half of that required in nature. There was no development in anthers cultured at the tetrad or the 1-celled microspore stage (Fig. 9).

The maximum development seen, both in *Allium cepa* and in *Rhoeo discolor*, was up to the formation of 1-celled microspores. Division of the microspore nucleus did not take place even in those cases where the anthers were excised and cultured at the tetrad or the 1-celled microspore stage. Attempts to achieve the division of the microspore nucleus in liquid cultures and in media supplemented with casein hydrolysate, yeast extract, and several of the amino-acids and vitamins also failed.

To the best of my knowledge there seems to be no other record of the cultivation of excised anthers with such a high degree of survival as reported here with the help of ribonucleic acid.

TABLE II

Development of anthers, excised at leptotene-zygotene (LZ) and at diplotene-diakinesis (DD), of Rhoeo discolor in White's medium with RNA additions. Conventions as for Table I

RNA mg./l.	Days	Excised at	Undeveloped	LZ	DD	MI	Dy	MII	Td
5	1	LZ	5	—	95	—	—	—	—
		DD	14	—	—	—	47	23	16
	2	LZ	—	—	100	—	—	—	—
		DD	11	—	—	—	38	—	51
10	1	LZ	—	—	58	12	30	—	—
		DD	—	—	4	—	38	35	23
	2	LZ	—	25	—	—	42	18	15
		DD	—	—	12	—	14	—	74
25	1	LZ	—	4	16	—	80	—	—
		DD	—	—	—	—	—	—	100
	2	LZ	—	3	—	—	76	—	21
		DD	all 1-celled microspores						
50	1	LZ	—	—	100	—	—	—	—
		DD	—	—	12	—	60	—	28
100	1	LZ	degenerating						
		DD	—	—	60	—	40	—	—

DISCUSSION

Although there is some correlation, as shown by Erickson (1947, 1948) and Taylor and McMaster (1954), between the length of the bud and the meiotic stage of its anthers, the determination of the meiotic stage on the basis of bud or anther length is not always accurate (*see also* Linskens, 1956*b*).

The problem of achieving the mitosis of the microspore nucleus *in vitro* has baffled several investigators. Taylor (personal communication) did not succeed in this with *Lilium* anthers even after the addition of kinetin and gibberellic acid. Linskens (personal communication) reported that the division of the microspore nucleus did not take place *in vitro*. He has, however, shown that there are some nucleotides in the tapetum at the beginning of the microspore division (these may in some way be responsible for the division of the microspore nucleus).

It has been shown by Taylor and McMaster (1954) and Moses and Taylor (1955) that DNA synthesis takes place at three specific stages in the development of pollen. These stages are: (i) preleptotene, (ii) shortly before the division of the microspore nucleus, and (iii) shortly after microspore mitosis, in the generative nucleus. Since all my attempts (Vasil, 1958) to induce the division of the microspore nucleus *in vitro* failed, I thought it worth while to use DNA, because just before the microspore mitosis there is synthesis of DNA in anthers developing in nature. However, while DNA and RNA failed to induce a division of the microspore nucleus, both supported the development of the excised anthers up to the formation of tetrads or even 1-celled microspores. In both *Allium cepa* and *Rhoeo discolor*, the best development of the anthers and the highest degree of survival was achieved in White's medium supplemented with RNA (50 and 25 mg./l. respectively). In *R. discolor* the time taken by anthers at diplotene-diakinesis to form tetrads *in vitro* was reduced by 50 per cent. Tetrads were formed in 24 hours, whereas in nature the time required for the same is 48 hours.

In tobacco pith tissue, Silberger and Skoog (1953) have shown a 'correlation of cell division with DNA increase at low concentrations of IAA'. Sharma and Sen (1954) and Hildebrandt *et al.* (1957) have reported an increase in cell divisions and growth with the use of RNA. The latter have concluded that the growth of marigold and tobacco tissues was 'strikingly stimulated by both filtered and autoclaved RNA at concentrations of 400 and 4,000 mg. per litre, while DNA has no effect at low concentrations and was strikingly inhibitory with 4,000 mg. per litre' (in the present investigation also RNA proved more useful than DNA).

Plaut (1953) suggested that 'meiosis is dependent upon a supply of materials secreted by the cells surrounding the developing microsporocytes or spermatocytes'. The fluid in which the microspores are bathed (periplasmodium) is derived from the dissolution of tapetal cells and of microspores which fail to complete development, and both of these are very rich in RNA. Therefore, it seems a safe inference that 'the anther fluid is the source of the nucleic acids

which accumulate in the cytoplasm of microspores' (Painter, 1943). Further, the supply of RNA from the follicular cells (comparable to the tapetum) to the germ cells (comparable to the microspore mother cells) seems to be very widespread in animals (Montalenti *et al.*, 1950). It has been suggested by Montalenti *et al.* (1950) that 'a large amount of RNA is the cause, or at least one of the causes of the starting of meiosis'. It may be pointed out here that the accumulation of RNA in the maturing pollen grains is needed for the extremely rapid synthesis of new materials which accompany pollen tube growth.

ACKNOWLEDGEMENTS

It is a pleasure to express my thanks and gratitude to Professor P. Maheshwari for his keen interest in the progress of this work and his valuable advice. Thanks are due to Professor J. H. Taylor (Columbia University, New York 27, N.Y., U.S.A.) for going through the manuscript, and to Professor Dr. H. F. Linskens (R.K. Universiteit, Nijmegen, The Netherlands) and Professor A. H. Sparrow (Brookhaven National Laboratory, Upton L.I., N.Y., U.S.A.) for their valuable suggestions.

LITERATURE CITED

Cooper, D. C. (1952). The transfer of desoxyribonucleic acid from the tapetum to the microsporocytes at the onset of meiosis. *Amer. Nat.* **86**, 219.

Erickson, R. O. (1947). Respiration of developing anthers. *Nature, Lond.* **159**, 275.

—— (1948). Cytological and growth correlations in the flower bud and anther of *Lilium longiflorum*. *Amer. J. Bot.* **35**, 729.

Hildebrandt, A. C., Riker, A. J., and Muir, E. (1957). Growth *in vitro* of marigold and tobacco tissue with nucleic acids and related compounds. *Plant Physiol.* **32**, 231.

Linskens, H. F. (1956*a*). Physiologie der Fortpflanzung und Sexualität. *Fortsch. Bot.* **18**, 329.

—— (1956*b*). Physiologische Untersuchungen zur Reifeteilung. I. Mitteilung. Über die Änderung einiger physiologischer Zustandsgrössen während der Pollenmeiose und Pollenentwicklung von *Lilium henryi*. *Ber. dtsch. bot. Ges.* **69**, 353.

—— (1958). Physiologische Untersuchungen zur Reifeteilung. II. Mitteilung. Über die Änderung des Nukleinsäurengehaltes während der Pollenmeiose und Pollenentwicklung von *Lilium henryi*. *Acta bot. Neerl.* **7**, 61.

Montalenti, G., Vitagliano, G., and De Nicola, M. (1950). The supply of RNA to the male germ cells during meiosis in *Asellus aquaticus*. *Heredity*, **4**, 75.

Moses, H. J., and Taylor, J. H. (1955). Desoxypentosenucleic acid synthesis during microsporogenesis in *Tradescantia*. *Exp. Cell Res.* **9**, 474.

Ogur, M., Erickson, R. O., Rosen, G. U., Sax, K. B., and Holden, C. (1951). Nucleic acids in relation to cell division in *Lilium longiflorum*. *Exp. Cell Res.* **2**, 73.

Painter, T. S. (1943). Cell growth and nucleic acids in the pollen of *Rhoeo discolor*. *Bot. Gaz.* **105**, 58.

Plaut, W. S. (1953). DNA synthesis in the microsporocytes of *Lilium henryi*. *Hereditas*, **39**, 439.

Sharma, A. K., and Sen, S. (1954). Induction of division through nucleic acid treatment. *Caryologia*, **6**, 151.

Silberger Jr., J., and Skoog, F. (1953). Changes induced by indoleacetic acid in nucleic acid contents and growth of tobacco pith tissue. *Science*, **118**, 443.

Sparrow, A. H., Moses, M. J., and Steele, R. (1952). A cytological and cytochemical approach to an understanding of radiation damage in dividing cells. *Brit. J. Radiol.* **25**, 182.

—— Pond, V., and Kojan, S. (1955). Microsporogenesis in excised anthers of *Trillium erectum* grown on sterile media. *Amer. J. Bot.* **42**, 384.

Steil, W. N. (1950). Some evidences for the interaction of tapetal and sporogenous cells in certain vascular plants. *Bot. Gaz.* **111**, 300.

TAYLOR, J. H. (1950). The duration of differentiation in excised anthers. *Amer. J. Bot.* **37**, 137.
—— (1953). Autoradiographic detection of incorporation of P^{32} into chromosomes during meiosis and mitosis. *Exp. Cell Res.* **4**, 169.
—— and MCMASTER, R. D. (1954). Autoradiographic and microphotometric studies on desoxyribosenucleic acid during microgametogenesis in *Lilium longiflorum*. *Chromosoma*, **6**, 489.
VASIL, I. K. (1957*a*). Effect of kinetin and gibberellic acid on excised anthers of *Allium cepa*. *Science*, **126**, 1294.
—— (1957*b*). Effect of kinetin and gibberellic acid on excised anthers of *Allium cepa*. *Phytomorphology*, **7**, 138.
—— (1958). *The cultivation of excised anthers and the culture and storage of pollen grains*. Ph.D. Thesis, Univ. of Delhi, Delhi, India.
WALKER, G. W. R. (1957). Observations of divisions in living pollen mother cells and pollen grains of *Tradescantia*. *Proc. Genet. Soc. Canada*, **2**, 18.

Respiratory Mechanisms and the Changes of Glutathione and Ascorbic Acid in Germinating Peas

S. P. SPRAGG[1] AND E. W. YEMM

Department of Botany, University of Bristol

Received 10 October 1958

SUMMARY

The respiration of pea seeds during the early stages of germination was studied by manometric methods. From analyses of the germinating seeds estimates were obtained of the oxidized and reduced forms of glutathione and ascorbic acid. Thus it was possible to explore the relationship between these oxidation-reduction systems and the resumption of respiratory activities in the dormant seed.

Several lines of evidence indicate that partial anaerobiosis is an important feature of metabolism during the first phases of germination in air. The intact testa, acting as a barrier to diffusion, limits the uptake of oxygen by the seed. Splitting of the testa by the development of the embryo leads to rapid changes in the mechanism and rate of respiration.

An increase in the relative proportions of the reduced forms of both glutathione and ascorbic acid is associated with the establishment of anaerobic conditions in the seed. This and other available evidence indicate that the glutathione-ascorbic acid carrier system plays only a minor part in aerobic oxidations during the early stages of germination. The possibility that glutathione has an important influence on the activation of enzymes in the first phase of germination is briefly considered.

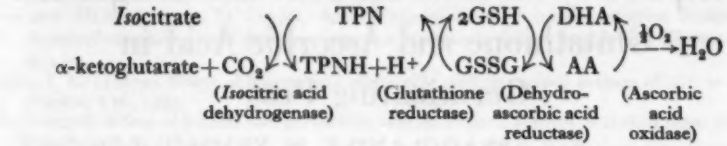
INTRODUCTION

THERE are many indications that glutathione and ascorbic acid take an active part in the metabolism of germinating peas. In the early stages of germination, both of these constituents undergo rapid changes, which strongly suggest that they are closely concerned with the resumption of metabolic activities in the dormant seed. Firket and Comhaire (1929) and Vivario and Lecloux (1930) found that sulphydryl compounds were formed within a few hours of supplying water to the seed; this was later confirmed by Hopkins and Morgan (1943), who isolated pure glutathione from moistened pea meal. Similarly, Robertson (1947) has shown that ascorbic acid is present in the seed in only small quantities but increases rapidly during the first 48 hours' germination.

More recently, evidence has been obtained that glutathione and ascorbic acid may act as respiratory carriers, particularly during the early stages of germination. With enzymic preparations from pea seeds, Mapson and Goddard (1951) were able to demonstrate the oxidation of intermediates of the tricarboxylic acid cycle, such as *iso*citrate and malate, by way of triphospho-

[1] Present address: National Vegetable Research Station, Wellesbourne, Warwick.

pyridinenucleotide (TPN), glutathione (GSH), and ascorbic acid (AA), as outlined below:



A study of the activity of this carrier system by Mapson and Moustaffa (1956) indicated that about one-quarter of the total respiration of the young seedling may be mediated by glutathione and ascorbic acid.

The experiments described in this paper were undertaken in order to explore the relation between the changes of glutathione and ascorbic acid and the respiration of germinating peas. Chief attention was given to the early stages of germination which mark the transition from a dormant condition. The seeds were germinated under controlled conditions of water-supply, aeration, and temperature so that the gradual resumption of oxygen uptake and carbon dioxide production could be measured. At suitable intervals samples of seeds were analysed and estimates were obtained of both the oxidized and reduced forms of glutathione and ascorbic acid. In this way it was possible to study the relationship between the oxidation-reduction systems and the respiratory activity in the young seedling.

EXPERIMENTAL METHODS

Seeds and germination. All the experiments were carried out with round-seeded peas (*Pisum sativum*) of the variety 'Meteor'. Four different samples of seeds were used; three of them were obtained commercially and the fourth was harvested from a normal garden crop in 1953.

Before germination, the seeds were sterilized by shaking them vigorously for 15 minutes in a solution of calcium hypochlorite, prepared by the methods described by Wilson (1915) and Bishop (1945). After washing with five changes of sterile distilled water, the seeds were aerated rapidly in distilled water at 22±1° C. in sterilized tubes fitted with sintered glass bubblers. The air stream was passed through cotton-wool filters before entering the bubblers. Under these conditions, germination occurred rapidly, 95–100 per cent. of the seeds showing radicles after 30–36 hours. The distilled water was replaced every 12 hours and no growth of micro-organisms was apparent as judged by the turbidity of the water. With the equipment available it was possible to germinate batches of 500 seeds as described above. Replicate samples, usually consisting of about 100 seeds, were taken at intervals for analysis of GSH and AA and for measurements of respiration. The zero time for all experiments was taken to be the time of sterilization with hypochlorite solution.

Extraction of soluble constituents. Two solvents, 2·5 per cent. w/v sulphosalicylic acid and 4 per cent. w/v metaphosphoric acid, were tested for extracting AA and GSH. No significant difference could be detected between

the values for AA obtained with the two extractants, but slightly higher values for GSH were obtained with sulphosalicylic acid. In view of its advantages for estimating GSH by enzymic methods (Woodward, 1935), sulphosalicylic acid was used for routine analyses, the extraction being carried out as follows. The sample of seeds (usually 20) was chilled to 1° C. in a mortar for 15 minutes and 10 ml. of ice-cold 5 per cent. sulphosalicylic acid added, followed by rapid grinding to a homogeneous paste. After allowing to stand for 15 minutes at 1° C., 10 ml. of chilled distilled water was added with a further thorough grinding. The extract was allowed to stand for 45 minutes and then centrifuged at 5,000 g. for 15 minutes in a cold room at 5° C. Estimations of GSH, GSSG, AA, and DHA in the clear, pale yellow extract were carried out immediately and were normally completed within 1–2 hours of preparing the extract.

In calculating the results, allowance was made for the water content of the seeds; the total volume of the extract was assumed to be that of the solvent added together with the water of the peas. The efficiency of extraction was tested by comparing it with three successive extractions: it was found that 90–95 per cent. of the total GSH was recovered in a single extraction by the method described above.

Most of the estimates of GSH and AA are based on the extraction of duplicate samples of 20 or more seeds. The agreement between the duplicate samples was nearly always within ± 10 per cent. of the mean.

Estimations of glutathione. A method similar to that of Grunert and Phillips (1951) was used for estimating GSH colorimetrically by means of the nitroprusside reaction. A suitable aliquot of the extract was added to 5 ml. of saturated NaCl in a colorimeter tube, and the total volume adjusted to 7 ml. with 2·5 per cent. w/v sulphosalicylic acid. After mixing, 1 ml. 0·067 M. sodium nitroprusside solution was added, followed immediately by 1 ml. of cyanide-carbonate solution (1·5 M. Na_2CO_3 and 0·067 M. KCN). The red colour was measured in a photoelectric colorimeter (Evans Electroselium Ltd.) with a green filter (Ilford No. 624) having maximum transmission at 520 mμ. Colorimeter readings were taken exactly 30 and 60 seconds after adding the cyanide-carbonate reagent. A blank determination, in which the extract was replaced by 2 ml. of sulphosalicylic acid, was carried out with the estimates.

The oxidized disulphide, GSSG, was estimated after reduction on a stirred mercury cathode by the method of Dohan and Woodward (1939). Treatment for 15 minutes at a current density of 2·5–3·1 mA/cm.^{2} was found to be adequate for complete reduction of GSSG or cystine. After reduction, an aliquot of the extract was used for estimation by nitroprusside as above.

The colour intensity developed by the reaction of GSH with nitroprusside is sensitive to temperature and varies slightly with the age of the nitroprusside solution. It was necessary, therefore, to prepare a calibration curve for each set of analyses. Pure glutathione (Distiller's Co.), freshly dissolved in 2·5 per cent. sulphosalicylic acid, was used for this purpose.

The nitroprusside reaction has the disadvantage that it is not specific for GSH; cysteine and hence, after reduction, cystine give a colour indistinguishable from that of GSH and of comparable intensity on a molar basis. However, the colour with cysteine is much less stable than that with GSH, so that an indication of the presence of the free amino-acid can be gained by measuring the rate of colour degradation, when extracts are treated under comparable conditions. The colour measurements made after 30 and 60 seconds were used for this purpose and strongly suggested that the sulphydryl of seedling extracts was mainly present as GSH.

A more critical test of the identity of the sulphydryl was made by means of the glyoxalase method of Woodward (1935), which is more specific for GSH. The source of glyoxalase apoenzyme was an acetone-dried preparation of yeast, washed three times with ten times its weight of distilled water. The conversion of methylglyoxal to lactic acid was measured manometrically at 25° C. with the precautions given by Woodward (1935). Activation of the apoenzyme by pure GSH was almost linear over a concentration range of 10–100 μg. GSH/ml. A three-point calibration was carried out with each series of determination; duplicate estimates usually agreed within $\pm 8{\cdot}0$ per cent. The mean of a number of colorimetric analyses exceeded the corresponding glyoxalase value by 7·5 per cent. It is probable, therefore, that nitroprusside over-estimates the quantity of GSH in the extracts. However, these data, together with the direct isolation of GSH from the seeds by Hopkins and Morgan (1943), indicate that the sulphydryl compounds in the extracts consist largely of GSH.

Estimation of ascorbic acid. Free AA was estimated in the extracts by titration with dichlorophenolindophenol freshly dissolved in distilled water so that 1 ml. was equivalent to approximately 75 μg. AA. The dye was standardized against a freshly prepared solution of AA in 2·5 per cent. sulphosalicylic acid. Total AA, including DHA, was estimated after reduction with H_2S. The extract was adjusted to pH 5·3 and a rapid stream of H_2S passed through it for 15 minutes, followed by standing for a further 30 minutes in a stoppered flask. After acidifying with 5N. HCl, H_2S was removed by bubbling N_2 through the solution for 15 minutes before titrating against the dye. For both of the above determinations reagent blanks were carried out at the same time.

Tests were made for the presence of other reducing substances which may interfere in the estimation of AA. Using the method described by Lugg (1942), it was found that relatively little interference occurred in the estimates of free AA in the extracts; it rarely exceeded 10 per cent. of the total titration, as was also found with pea seeds by Mapson (1943). However, tests carried out by the method of Huelin (1949) indicated that considerable quantities of reducing substance other than AA were present in extracts after treating them with H_2S. Particularly in the early stages of germination interfering substances may represent a substantial part of the increase in reducing power. In two of the experiments, therefore, separate determinations were made of

interfering substances by the method of Huelin (1949), so that the estimates of DHA could be corrected in this way.

Measurement of respiration. Gaseous exchanges were measured manometrically by the method of Warburg, as described by Dixon (1943). Flasks of conventional design, but of about 50 ml. capacity, were used to accommodate samples of 10–20 seeds. Water (usually 3 ml.) was added to the main compartment to avoid drying of the seeds. Carbon dioxide production was estimated by using replicate samples and omitting KOH from the central well of some of the Warburg flasks. In one of the experiments triplicate samples were used for measurements of both O_2 uptake and CO_2 production at different stages of germination. The agreement between comparable samples was usually within ± 10 per cent.; in a typical experiment with seeds germinated for 25 hours, six estimates of the respiratory quotient in air gave values of 2·2–3·0 with a mean of 2·7 and standard error $\pm 0{\cdot}2$.

Measurements of both O_2 uptake and CO_2 production were sometimes made on the same sample of seeds. The mean rate of oxygen uptake was first estimated over a period of about 2 hours and the KOH was carefully removed from the central well and replaced by 3N. H_3PO_4. Measurements were then continued and, assuming a steady rate of O_2 uptake, the production of CO_2 was calculated from the manometric data. Comparison of this method with that in which replicate samples were used gave satisfactory agreement. A parallel determination of the R.Q. of seeds after 25 hours' germination gave a value of 3·2 compared with a mean value of $2{\cdot}7 \pm 0{\cdot}2$ by the alternative method described above.

In some of the experiments respiration was measured in air and in pure oxygen obtained from a gas cylinder. Most of the experiments were carried out at 25° C. but for lower temperatures the Warburg apparatus was operated in a cold room, so that regulation of the water bath at 4·5° C. was possible.

Determinations of volume and moisture content. In order to measure the gaseous exchanges manometrically, the volume of the seeds and seedlings was determined with a volumenometer, constructed from the design of Weatherley (1950) giving results accurate within 1·5 per cent. Moisture content of the peas was determined by drying them for 24 hours at 105° C., followed by cooling over concentrated sulphuric acid.

EXPERIMENTAL RESULTS

Changes of seed constituents during germination

Water content and volume of seed. Determinations of the water content and volume of the seed were made at different stages of germination in four of the experiments. A rapid uptake of water and an increase in volume occurred during the first 12 hours under these conditions of germination. Thereafter a much slower increase of water content and volume took place, associated with growth of the embryo. Some typical results are shown in Fig. 1. In connexion with these measurements, an attempt was made to estimate the extent of the intercellular air spaces in the swollen seed. No significant difference

could be detected between the total volume of the seed, determined by displacement of water, and that estimated manometrically by means of the volumenometer. In addition, stripping off the testa and cutting across the cotyledons had no appreciable effect on the measurements by volumenometer, indicating that failure of the gas to equilibrate in the intercellular spaces of

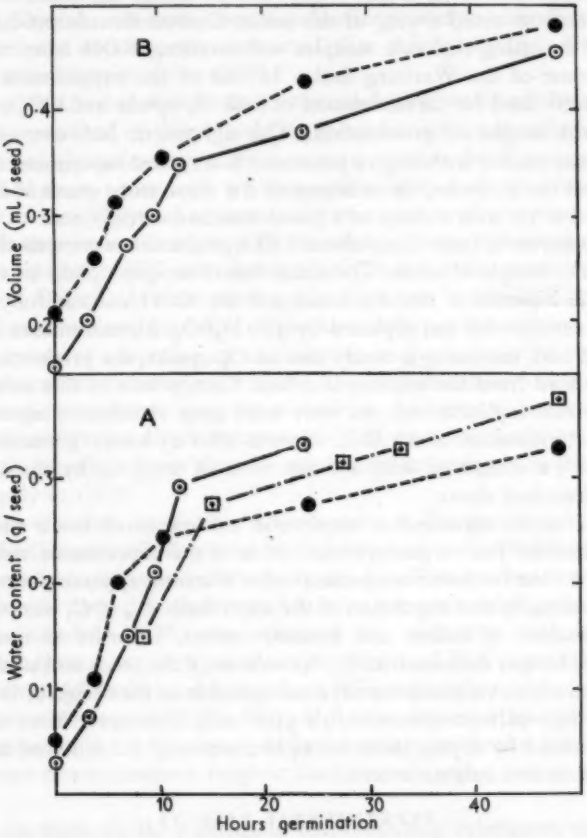


FIG. 1. *A*, the uptake of water and *B*, the increase in volume during the first 48 hours of germination: data obtained from three experiments with different batches of seeds.

the tissues of the seedling did not cause false readings of tissue volume by this method. Having regard to the accuracy with which these determinations were made, it is clear that at least up to 48 hours' germination intercellular air spaces in the swollen seed did not exceed 1·5 per cent. of the total volume of the seed and may be much less than this.

Changes of glutathione. The data drawn from all the experiments with dif-

ferent samples of seed have shown that a conversion of GSSG to GSH occurred during the early stages of germination. A typical example of the results is given in Fig. 2. Considerable increases of GSH were generally observed after 6 hours and the quantity present in the seedling reached a maximum after 24–48 hours and then declined. These changes could be

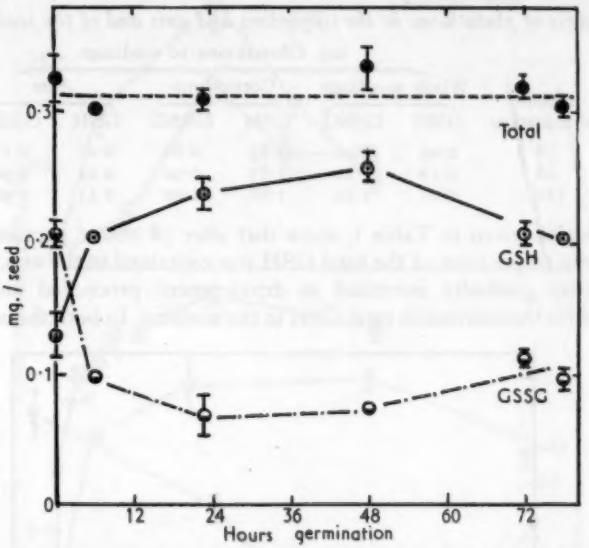


FIG. 2. The changes of reduced glutathione (GSH) and oxidized glutathione (GSSG) in seeds germinated for 72 hours. Most of the estimates are based on analyses of duplicate samples: the variation between duplicates is indicated by the vertical lines.

largely accounted for by the conversion of GSSG to GSH; no significant change in total GSSG+GSH could be detected during the first 48 hours' germination in any of the five experiments in which analyses were made. With more prolonged germination a gradual increase was observed, mainly in the form of GSSG.

The extent of the conversion of GSSG to GSH varied considerably in the different samples of seed which were investigated. The seed freshly harvested from a garden crop contained most of the tripeptide in the reduced form, and there were correspondingly smaller charges of GSSG to GSH during germination. It seems probable that harvesting or storage conditions have an important influence on the relative proportions of the oxidized and reduced forms of GSH in the mature seed. The samples of seed harvested in 1952 and in 1953 both showed a gradual fall in the amount of GSH when they were stored in an air-dry condition. In addition, the highest levels of GSSG were found in seeds which had been stored for at least a year, suggesting that a slow conversion of GSH to GSSG may occur during storage.

Some further analyses of germinating seeds have shown that most of the GSH was contained in the cotyledons of the young seedling. In one of the experiments, seeds germinated for 78–120 hours were dissected into cotyledons and axis (radicle and plumule) and these were subjected to separate analysis.

TABLE I

Changes of glutathione in the cotyledons and axis and of the seedlings

Hours germination	mg. Glutathione/10 seedlings					
	Whole seedlings		Cotyledons		Axis	
	GSH	GSSG	GSH	GSSG	GSH	GSSG
78	2·05	0·91	1·85	0·80	0·20	0·11
96	2·12	1·20	1·87	0·90	0·25	0·30
120	2·21	1·42.	1·88	1·02	0·33	0·40

The results, given in Table I, show that after 78 hours' germination approximately 10 per cent. of the total GSH was contained in the axis, although the quantity gradually increased as development proceeded and largely accounted for the increase in total GSH in the seedling. In both the cotyledons

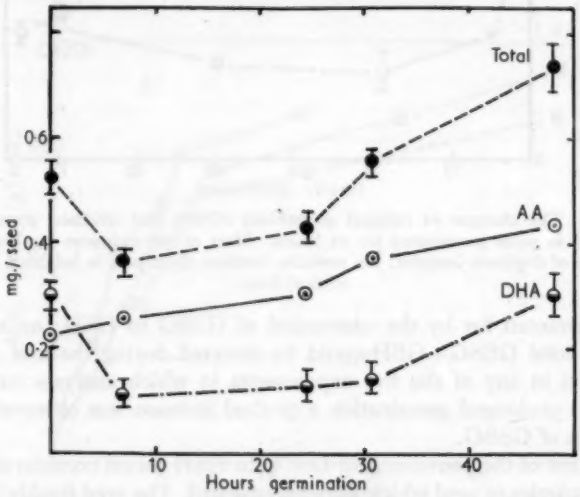


FIG. 3. The changes of ascorbic acid (AA) and dehydroascorbic acid (DHA) during the first 48 hours of germination. The variation between estimates in duplicate samples is indicated by vertical lines.

and axis there was evidence of a decrease in the ratio of GSH:GSSG in the later stages of germination.

Changes of ascorbic acid. In contrast with glutathione, total AA in the seeds showed marked changes during the first 48 hours of germination. Both of the experiments, in which correction could be made for interfering substances, agreed closely and the results of one of them are shown in Fig. 3. A decrease

of total AA occurred during the first 12–24 hours. This was followed by a rise to a value considerably higher than that of the dry seed, as was also observed by Robertson (1947).

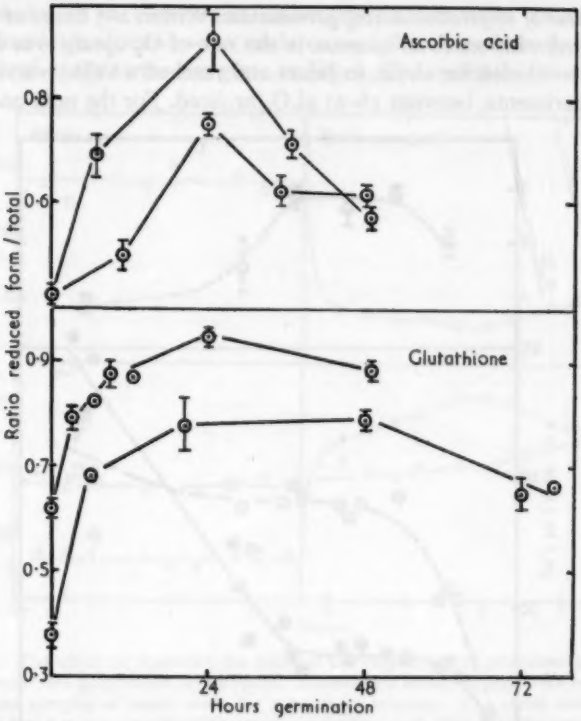


FIG. 4. The relative proportion of the reduced forms of glutathione and ascorbic at different stages of germination. Data drawn from experiments on two different batches of seeds.

The initial loss of total AA is attributable to a decrease in DHA content; AA rises steadily throughout germination and both constituents contribute to the increase observed after 24 hours.

A consistent feature of the changes of both AA and GSH is the increase in the relative proportions of the reduced forms which reach a maximum after about 24 hours' germination. This is shown in Fig. 4 in which the ratios of the reduced to total GSH and AA are given at different stages of germination. The extent of the changes of GSH varied considerably in different experiments. In the samples of freshly harvested seeds most of the GSH was present in the dry seeds as the reduced form, but even here an appreciable increase, at the expense of GSSG, was observed during the first 24 hours of germination. It is clear that the sharp rise in the ratio of AA to total AA is not due

simply to an alteration in equilibrium between oxidized and reduced forms, since there is a marked change in the total AA in the seedling at this time.

Respiration of the germinating seeds

The course of respiration during germination. Within 2–3 hours of supplying the dry seed with water an increase in the rate of O_2 uptake was detectable. The rise continued for about 10 hours and reached a value, varying in different experiments, between 16–21 μl.O_2/hr./seed. For the next 20–24 hours

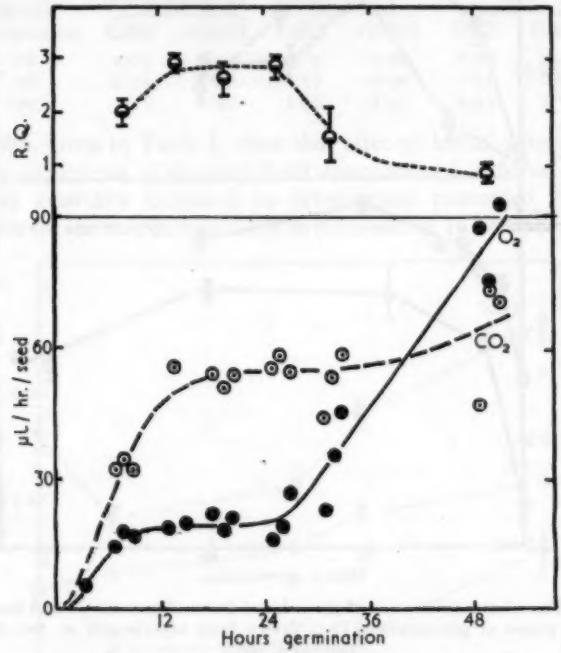


FIG. 5. The rate of respiration and respiratory quotient (RQ) during the first 50 hours of germination. Values of the respiratory quotient were calculated from triplicate measurements of O_2 uptake and CO_2 production: mean values are plotted and the range of variation shown by vertical lines.

no further significant changes in the rate of oxygen uptake were detected, but after 30–36 hours there was a further sharp increase. The results of a typical experiment in which the exchanges of both oxygen and carbon dioxide were measured are shown in Fig. 5. Similar results were obtained with all of the batches of seeds which were investigated in this way.

The influence of the testa on respiration. It was observed consistently that the rise in respiration after 30–36 hours' germination corresponded closely in time with splitting of the testa by the elongating radicle of the seedling. Several experiments were therefore carried out in which the effects of arti-

ficially removing the testa were investigated. By careful dissection the testa could be stripped from the swollen seed without injury to the embryo, and it was thus possible to compare the respiration of intact seeds with that of seeds from which the testas were removed.

The results of an experiment, in which measurements of oxygen and carbon-dioxide production were made on samples of seeds both before and after removal of the testas, are given in Fig. 6. The removal of the testa from

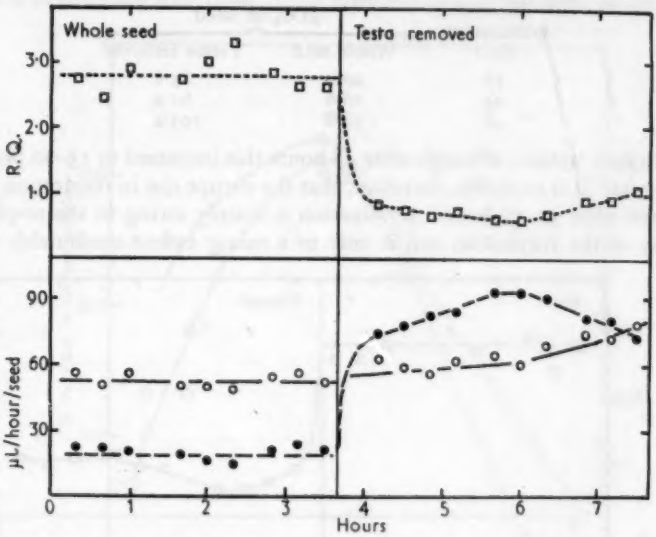


FIG. 6. The effect of removing the testa on the respiration of germinating seeds. The seeds were germinated for 18 hours. Manometric measurements were made on duplicate samples of intact seeds for 3 hours 40 minutes. The testas were then removed and measurements of O_2 uptake and CO_2 production were resumed after re-equilibration.

these seeds, which had germinated for 18 hours, caused a rapid increase in oxygen uptake to a value 3–4 times higher than that of the intact seeds. Carbon dioxide production, on the other hand, was at first much less affected and, as a result, the R.Q. fell to a value below one and then gradually rose to unity about 3 hours after the testas had been removed.

In other experiments O_2 uptake was measured on samples of seeds both before and after removal of the testa; the results obtained with seeds germinated for 18, 24, and 48 hours are summarized in Table II. Both of the samples, which were investigated before splitting of the testa had occurred, showed large increases in the rate of oxygen uptake. After 48 hours the testa was ruptured but still partially covering the cotyledons; its removal at this stage had a smaller effect on the respiration of the seed.

During the course of these experiments estimates were obtained of the

contribution made by different parts of the embryo to the respiration of the whole seed. Seedlings were dissected by removing the testas and separating the axis (plumule and radicle) by a razor cut at the base of each cotyledon. After 24 hours' germination the axis contributed less than 1 per cent. of the

TABLE II

Effect of removing the testa on respiration

Time of germination (hr.)	$\mu l.O_2/hr./seed$	
	Whole seed	Testas removed
18	20·4	85·6
24	18·6	61·2
48	79·8	103·2

total oxygen uptake, although after 48 hours this increased to 15–20 per cent. of the total. It is probable, therefore, that the abrupt rise in respiration which occurred after 30–36 hours' germination is mainly owing to the respiratory activity of the cotyledons and is only to a minor extent attributable to the

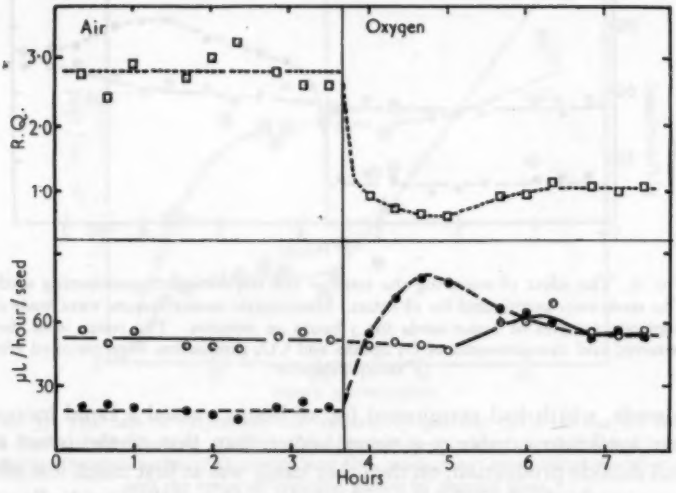


FIG. 7. Respiration of germinating seeds in air and in oxygen. The seeds were germinated for 18 hours. The rate of O_2 uptake and CO_2 production was measured by means of duplicate samples first in air and then in oxygen. The changes in the respiratory quotient are shown in the upper part of the figure.

development of the plumule and radicle. Measurements of the oxygen uptake of separated testas showed that they made a negligible contribution to the respiration of the seeds under these conditions. The possibility can therefore be eliminated that the activity of micro-organisms on the testa had an appreciable effect on the measurements of respiration.

The effects of oxygen tension on respiration. The effects of different oxygen

tensions on the respiration of the germinating seeds were studied with the object of investigating the extent to which the respiration of the seed may be limited by the supply of oxygen to the tissues. In most of these experiments the rate of respiration of the seeds in air was compared with that in pure oxygen; the results of an experiment in which replicate samples of seeds were investigated after 24 hours' germination are shown in Fig. 7.

It is apparent that the effects of the high oxygen tension have several features in common with those found after removal of the testa, as described

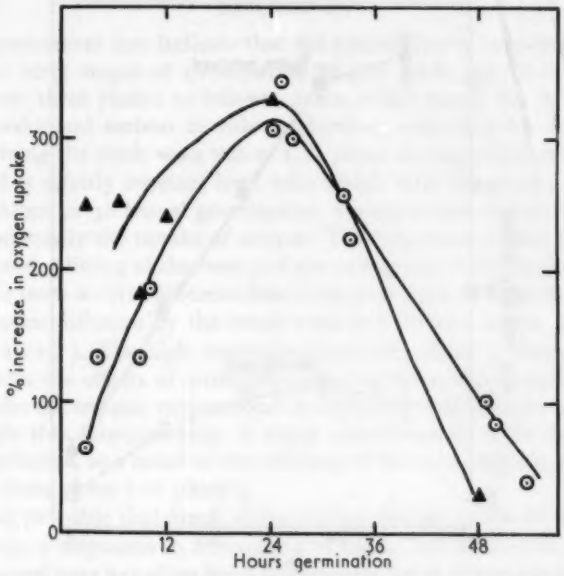


FIG. 8. The effect of high oxygen tensions on the rate of respiration at different stages of germination. Data drawn from experiments in which oxygen uptake was measured manometrically, first in air, and then in oxygen. The percentage increase in oxygen uptake was calculated from the mean rates observed over periods of 1 hour before and after transfer from air to oxygen. Different batches of seed were used for two series of experiments.

in the previous section. On transferring the seeds from air to pure oxygen there was a rapid increase in the rate of oxygen uptake, but a much smaller effect on the rate of carbon dioxide output. The R.Q. fell abruptly to a value considerably lower than 1·0. This was followed by a gradual fall in the rate of oxygen uptake and the R.Q. rose to a value near to unity after 2–3 hours in pure oxygen. The effects of supplying the seeds with pure oxygen on the rate of respiration varied at different stages of germination. In two of the experiments the rates of oxygen uptake of two sets of replicate samples were measured either in air or in pure oxygen during the first 48 hours of germination. The percentage increases in the rates of oxygen uptake in pure oxygen relative to

the rates in air are shown in Fig. 8. The relative rate of oxygen uptake increased markedly during the first 24 hours of germination to a value about three times greater than that in air. In the succeeding 24 hours the effects of oxygen became progressively smaller. The period of maximum effect coincided with the phase of relatively constant rate of respiration and high R.Q. in the intact seed (see Fig. 5).

The effect of temperature on the rate of respiration. Some further evidence of the influence of the testa on gaseous exchanges of the intact seed was obtained

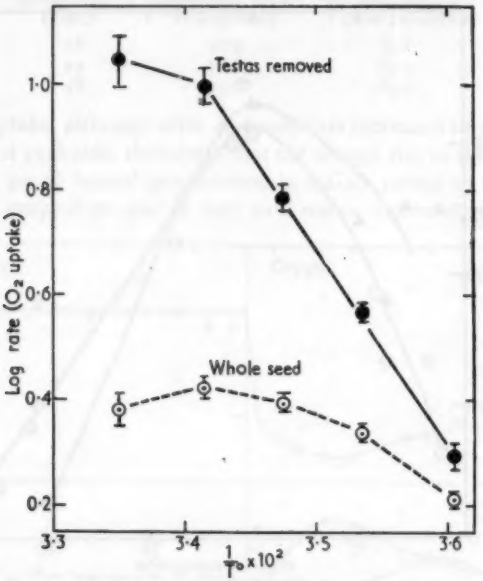


FIG. 9. The respiration of seeds with or without testas at different temperatures (for explanation see text).

from a study of the rate of respiration at different temperatures. Seeds were germinated under the standard conditions for 24 hours and then divided into two samples. From one of these the testas were removed by dissection, while the other consisted of intact seeds. The uptake of oxygen was measured in air at temperatures of 4·5, 10, 14·8, 20, 25·5° C. Duplicate samples were used for each series and mean rates were calculated from measurements extending over about an hour when the rate remained relatively constant.

At each temperature the mean rate of oxygen uptake by intact seeds was much lower than that of the comparable samples from which the testas had been removed. Analysis of the data has shown that up to a temperature of 20° C. the effects of temperature on the rate of respiration of seeds without testas agreed closely with that expected from the Arrhenius' equation for thermochemical reactions (Moelwyn-Hughes, 1933). This is demonstrated by

plotting log rate against the reciprocal of the absolute temperature, as shown in Fig. 9. Over the temperature range 4–20° C. there was a linear relation between the two functions; from the line of best fit an 'activation energy' of 17·1 K. cals. was calculated for the uptake of oxygen by seeds from which the testas had been removed. With intact seeds, on the other hand, no such linear relation occurred. There was a gradual decline in the increments in rate with increasing temperature, indicating a progressive limitation of the respiration of the seed by physical processes of gaseous diffusion across the testa.

DISCUSSION

The experimental data indicate that the resumption of respiratory activity during the early stages of germination of pea seeds can be conveniently analysed into three phases as follows: 1. An initial steady rise in the rate of oxygen uptake and carbon dioxide production, extending for 10–12 hours after supplying the seeds with water. 2. A phase during which respiration is maintained at a fairly constant level with a high R.Q. for about a further 20 hours. 3. After 30–36 hours' germination, a conspicuous rise in rate occurs, affecting especially the uptake of oxygen. The beginning of this phase coincides with the splitting of the testa and the emergence of the seedling radicle.

Evidence from several different directions converges to show that a limitation of gaseous diffusion by the intact testa is a decisive factor, particularly in phases 2 and 3. The high respiratory quotient, about 3 during phase 2, together with the effects of artificially removing the testa and exposure of the seed to different oxygen tensions and to different temperatures are all consistent with this interpretation. A rapid improvement of the facilities for gaseous exchange, as a result of the splitting of the testa, appears to mark the transition from phase 2 to phase 3.

It is very probable that much of the carbon dioxide produced by the seed during phase 2 originates in anaerobic processes. The production of ethanol in germinating peas has often been demonstrated (see, for example, Goksöyr *et al.* (1953)); from the present data it can be estimated that about two-thirds of the carbon dioxide may arise by alcoholic fermentation during the early stages. On the other hand, the respiratory mechanisms undergo a drastic change after the testa has split. Some of the experiments indicate that a powerful Pasteur effect operates to regulate glycolysis when supplies of oxygen are improved either by the splitting of the testa or by the exposure of the seeds to high oxygen tensions. The rate of carbon dioxide production is relatively little affected under these conditions (see Figs. 6 and 7) and it can be estimated that the rate of carbohydrate breakdown is reduced to about a half, assuming that alcoholic fermentation is replaced by aerobic oxidations. However, it is by no means certain that carbon dioxide production is a satisfactory measure of glycolysis, since fluctuations of the R.Q. suggest that the oxidation of highly reduced metabolites, such as ethanol, may take place during the transition from anaerobic to aerobic conditions.

In a recent study of alcohol dehydrogenase in peas, Goksöyr *et al.* (1953)

have found that the activity of this enzyme rises to a maximum in the first two days of germination and then rapidly declines.

James (1953) has reviewed the evidence that anaerobiosis may be a normal feature of the physiology of seeds during the first stages of germination. In the present experiments with pea seeds it was shown by methods similar to those described by Burton (1950) that no mass movement of gas occurred through the intact testa. Within the compact structure of the seed, gaseous exchanges may be further restricted by the very small volume of the intercellular and other air spaces.

It is against this background of limited oxygen-supply and partial anaerobiosis that the changes of glutathione and ascorbic acids in the germinating seeds must be considered. A feature of both of these oxidation-reduction systems is their tendency to pass into a predominantly reduced condition during the first and second phases of germination, a tendency which reaches a maximum after about 24 hours. This corresponds with the time of most severe limitation of respiration by oxygen-supply, and strongly suggests that the oxidation-reduction systems closely reflect the anaerobic conditions in the seed. No overall synthesis of glutathione occurs during the first 48 hours and the reduction of GSSG to GSH takes place mainly in the cotyledons. Ascorbic acid, on the other hand, increases appreciably in amount and is probably synthesized during the early phases of germination.

Another factor, which may limit the extent to which the glutathione-ascorbic acid oxidation systems operate during the early stage of germination, may be the low activity of ascorbic acid oxidase. Mapson and Moustaffa (1956) have shown that the activity of this enzyme is not fully developed until after 3 days' germination. In addition to these developmental changes the low affinity of ascorbic acid oxidase for oxygen, discussed by Thimann *et al.* (1954) and by Bonner (1957), may reduce the efficiency of the system, relative to that of other terminal oxidases, at low oxygen tensions. The data at present available suggest that glutathione and ascorbic acid play only a minor part in the aerobic oxidations during the first phases of seed germination.

It is probable that glutathione has a wide influence on cell metabolism, extending beyond its action as an electron carrier in oxidation processes. Racker (1954) has shown that GSH acts as a prosthetic group in triosephosphatedehydrogenase. Furthermore it may activate many enzymes which depend for their activity upon sulphydryl groups (Barron, 1951). These and other broad aspects of the metabolism of glutathione have been reviewed in the symposium edited by Colowick (1954). The rapid formation of GSH in the first stages of germination may play an important part in the activation of enzymic systems during the transition from a dormant condition. In this connexion it is of interest that Ruge (1948) has shown that the germination of some seeds can be restored after prolonged storage by treatment with glutathione. Some of the observations recorded here suggest the gradual oxidation of sulphydryl compounds in the dormant seed may be one of the factors underlying the loss of viability.

ACKNOWLEDGEMENT

We are indebted to the Food Investigation Organization of the Department of Scientific and Industrial Research for financial and other assistance in this work.

LITERATURE CITED

BARRON, E. S. G. (1951). Thiol groups of biological importance. *Advances in Enzymology*, **11**, 201.

BISHOP, L. R. (1945). Second memorandum on barley germination. *J. Inst. Brewing*, N.S. **42**, 215.

BONNER, W. (1957). Soluble oxidases and their functions. *Ann. Rev. Pl. Physiol.* **8**, 427.

BURTON, W. G. (1950). Studies on the dormancy and sprouting of potatoes. I. The oxygen content of the potato tuber. *New Phytol.* **49**, 121.

COLOWICK, S. P. (1954). *Glutathione: proceedings of the symposium held at Ridgefield, Connecticut*. Academic Press, New York.

DIXON, M. (1943). *Manometric Methods*. Cambridge University Press.

DOHAN, J. S., and WOODWARD, G. E. (1939). Electrolytic reduction and determination of oxidised glutathione. *J. Biol. Chem.* **129**, 393.

FIRKET, J., and COMHAIRE, S. (1929). Recherches expérimentales sur la teneur en glutathion des pois au début de la germination. *Bull. Acad. Roy. Med. Belg.* **9**, 93.

GOKSÖYR, J., BOERI, E., and BONNICHSEN, R. K. (1953). The variation of ADH and catalase activity during the germination of green peas (*Pisum sativum*). *Acta Chem. Scand.* **7**, 657.

GRUNERT, R. R., and PHILLIPS, P. H. (1951). A modification of the nitroprusside method of analysis for glutathione. *Arch. Biochem.* **30**, 217.

HOPKINS, F. G., and MORGAN, E. J. (1943). Appearance of glutathione during the early stage of the germination of seeds. *Nature*, **152**, 288.

HUELIN, F. E. (1949). Investigations on the stability and determination of dehydroascorbic acid. *Australian J. Sci. Research*, B, **2**, 346.

JAMES, W. O. (1953). *Plant Respiration*. Oxford University Press.

LUGG, J. W. H. (1942). The use of formaldehyde and 2,6-dichlorphenolindophenol in the estimation of ascorbic acid and dehydroascorbic acid. *Australian J. Exp. Biol.* **20**, 273.

MAPSON, L. W. (1943). Vitamin methods: VI. Vitamin C in presence of reductone substances. *J. Soc. Chem. Ind.* **62**, 223.

—— and GODDARD, D. R. (1951). The reduction of glutathione by plant tissues. *Biochem. J.* **49**, 592.

—— and MOUSTAFFA, E. M. (1956). Ascorbic acid and glutathione as respiratory carriers in the respiration of pea seedlings. Ibid. **62**, 248.

MOELWYN-HUGHES, E. A. (1933). *Kinetics of reactions in solution*. Oxford University Press.

RACKER, E. (1954). Glutathione as a coenzyme in intermediary metabolism. (In *Glutathione: a symposium*, Colowick, S. *et al.*, eds.) Academic Press, New York.

ROBERTSON, R. N. (1947). The accumulation of vitamin C during germination in peas. *Australian J. Exp. Biol.* **25**, 41.

RUGE, U. (1948). Untersuchungen über keimungsfordernde Wirkstoffe. *Planta*, **35**, 297.

THIMANN, K. V., YOCUM, C. S., and HACKETT, D. P. (1954). Terminal oxidases and growth in plant tissues. III. Terminal oxidation in potato tuber tissue. *Arch. Biochem. Biophys.* **53**, 239.

VIVARIO, R., and LECLOUX, J. (1930). Sur la formation de glutathion au cours de la croissance. *Arch. Intern. Physiol.* **32**, 1.

WEATHERLEY, P. E. (1950). A convenient volumenometer for biological work. *J. Exp. Bot.* **1**, 244.

WILSON, J. K. (1915). Calcium hypochlorite as a seed sterilizer. *Amer. J. Bot.* **2**, 420.

WOODWARD, G. E. (1935). Glyoxalase as a reagent for the quantitative microestimation of glutathione. *J. Biol. Chem.* **109**, 1.

The Boron Requirement of Flax Roots Grown in Sterile Culture

T. F. NEALES

The Botany School, University of Melbourne, Carlton, N. 3, Victoria, Australia

Received 2 March 1959

SUMMARY

Methods are described which eliminate boron contamination in the culture medium.

Excised flax roots, grown in sterile culture, are shown to have a boron requirement which is satisfied by 0·05 p.p.m. boron. Root growth stopped after 48 hours in media lacking boron. The boron requirement of excised roots is similar to that of whole flax seedlings.

It is concluded that the observed restriction of root growth in media lacking boron is not connected with the movement of sugars into flax root cells.

INTRODUCTION

ALTHOUGH boron is an essential element for the growth of vascular plants, and also of some algae (Krauss, 1958), its requirement for excised roots grown in sterile culture has not been demonstrated (Glasstone, 1947; Hannay and Street, 1954; Street, 1957). This is probably due to the use by earlier workers of borosilicate glassware and glass-distilled water.

One of the most striking effects of boron deficiency in vascular plants is the abrupt inhibition of the growth of meristems of root and shoot. Roots are particularly sensitive to the absence of boron; bean radicles, attached to their cotyledonary food reserves, need a supply of boron in the medium if root elongation is to proceed for more than 48 hours (Whittington, 1957).

Gauch and Dugger (1953, 1954) and Sisler, Dugger, and Gauch (1956) have postulated that 'boron plays a specific role in the translocation of sugars' in plants. Extending this concept, they (1953, 1954) have further suggested that the growth of root and shoot meristems of boron-deficient plants is restricted by sugar deficiency.

This paper describes a study on the boron requirement of excised flax roots grown in a sterile medium containing sucrose. This involved the development of procedures eliminating the use of glass culture dishes and glass-distilled water. The effect of boron concentration on the uptake of ^{14}C sucrose by flax roots was also examined with the object of testing the hypotheses of Gauch and Dugger (loc. cit.).

METHODS AND MATERIALS

Flax seeds (var. Ventnor), sterilized in a ethanol-hydrogen peroxide mixture (Langridge, 1957), were transferred aseptically to moist strips of No. 3

Whatman filter-paper in sterilized 6″×1″ boiling tubes plugged with cotton-wool. After 48 hours' germination at 27° C., 10 mm. root tips were transferred aseptically to the culture solutions.

The composition of the growth medium is given in Table I. It is a modification of that used by Bonner and Devirian (1939). Iron was supplied as FeEDTA in the molar ratio Fe:EDTA of 1:2·45 (Sheat, 1956, quoted by Street, 1957).

TABLE I

The composition of the medium used for the growth of excised flax roots, cited as mg./litre. pH after autoclaving 4·95±0·06. *Together with boron as specified in text*

$Ca(NO_3)_2$	336	Cu	0·005
KH_2PO_4	60	Zn	0·005
$MgSO_4.7H_2O$	42	I	0.025
Fe (as FeEDTA)	1·0	Na	0·010
Mn	0·100	Thiamine	0·10
Mo	0·002	Sucrose	40,000

The three compounds supplying the macronutrients were freed from boron contamination by repeated recrystallization (Hewitt, 1952, p. 191). The micronutrients and iron were supplied from B.D.H. reagents, A.R. grade. Sucrose was purified by making use of the fact that in alkaline solution boric acid combines with mannitol to form a negatively charged complex (Böeseken, 1949). Mannitol was added to a 15 per cent. sucrose solution at a concentration of 0·001 M. This solution was then made alkaline (pH 9·5) with NaOH and twice passed over an anion exchange resin (Deacidite, FF) held in a polythene tube of a bed volume of 550 mls. Finally, the pH of the effluent was adjusted to 5·5 with $2N.H_2SO_4$. The concentration of this stock sucrosensolution was measured with a refractometer and it was stored at −20° C. in polythene containers. Mannitol (0·001M) was shown to be without effect on the growth of excised flax roots. In later experiments it was shown that B.D.H. sucrose (A.R. grade) was practically free of boron (Table III).

The water used throughout these experiments was once metal distilled, stored in polythene containers, and passed over a mixed-bed resin (Biodimineralite) before use. It was shown in preliminary experiments that pyrex-distilled water supplied approximately half the boron required for 3 weeks' normal growth of flax seedlings in nutrient culture (Table II).

50 ml. of the root-growth medium was added to 13 cm. diameter aluminium petri-dishes. Each of these was covered by a glass petri-dish lid. The medium was then autoclaved at 15 lb. pressure for 15 minutes. When cool, 10 μl. of a sterile solution of thiamine was added, making a final concentration of 0·1 mg. thiamine/l.; after which five 10 mm. root tips were transferred aseptically to each dish. It proved impossible to produce boron deficiency consistently in flax roots grown in pyrex petri-dishes, whether they were coated with a silicone film ('Sil-cote' SF 114, Anax Pty. Ltd., Melbourne) or not. Polished stainless steel and aluminium petri-dishes, however, were equally effective (Table III and Fig. 1).

TABLE II

The effect of distilled water from various sources, added to a boron-free culture medium, on the height and fresh weight of flax seedlings grown for 3 weeks

Source of distilled water	B added (p.p.m.)	Mean stem height (cm).	Fresh weight 10 plants (g).
Metal distilled	0	2·9±0·2	1·35
	0·5	18·7±0·5	9·02
Metal distilled, then passed over mixed-bed resin	0	3·1±0·2	1·30
	0·5	20·2±0·4	9·04
Once pyrex distilled water	0	10·9±0·3	3·73
	0·5	20·3±0·3	11·90
Once pyrex distilled (new still and condenser)	0	16·6±0·4	6·81
	0·5	20·9±0·7	11·51
Twice pyrex distilled	0	17·4±0·6	7·24
	0·5	20·2±0·7	11·89

TABLE III

The ability of various media and containers to support the growth of flax roots in the absence of an added boron supply

Treatment	p.p.m. boron supplied	Final mean length of 10 mm. root tip explants after 7 days' growth (mm).	Number of laterals formed per root.
Aluminium containers, standard medium.	0	21·4±1·5	0
	0·1	129·3±6·4	12
Stainless-steel containers, standard medium	0	17·5±1·1	0
	0·1	117·0±7·3	11
Aluminium containers, medium made up with twice pyrex-distilled water	0	47·5±3·2	3
	0·1	132·5±6·9	12
Pyrex petri-dishes, standard medium	0	61·3±15·5	4
	0·1	131·6±6·7	12
Aluminium containers, medium made up with unpurified sucrose, B.D.H., A.R. grade	0	27·3±1·1	1
	0·1	128·5±6·3	14

Cultures were maintained in the dark at 27°±0·1° C. Accurate daily measurements of root length were impossible to obtain on account of the opaque aluminium dishes used. Daily estimates of root length were made visually. Root lengths were accurately measured after 7 days' growth by removing the roots and placing them on mm. ruled graph paper.

The medium supported the active growth of flax roots for 10 weeks, over which time a growth-rate of 15–22 mm. per day was maintained. This is similar to that recorded by Bonner and Devirian (1939). However, the

magnitude of the growth-inhibitory effect of boron deficiency was such that experiments were only normally continued for 7 days.

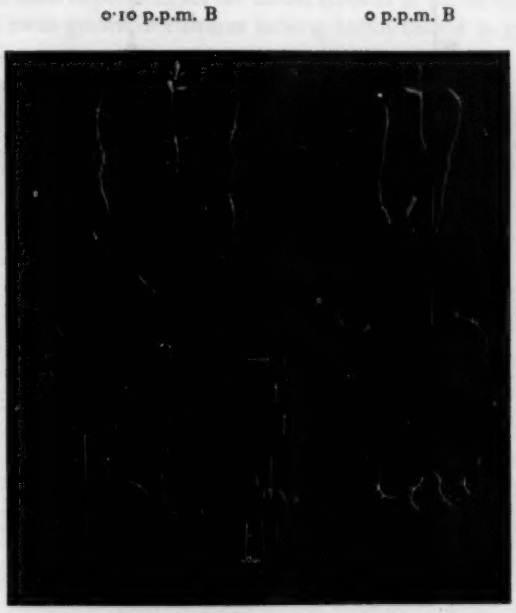

0·10 p.p.m. B
0 p.p.m. B
Flax seedlings grown in nutrient solution
Excised flax roots grown in pyrex petri-dishes
Excised flax roots grown in aluminium dishes

FIG. 1. The effect of boron on the growth of flax seedlings and excised flax roots after 6 days' culture.

Autoradiographs were made of roots after drying them between blotting-paper for 1 week, with light pressure applied; after which they were placed in contact with Kodak duplitized X-ray film for a further week. The presence of ^{14}C sucrose in the roots was verified by spotting an ethanolic extract of the roots on 1″ strips of Whatman No. 3 paper. The chromatograms were run in an ethyl acetate/pyridine/water (80:20:10 v/v) (White and Secor, 1953) solvent for 19 hours. These strips were then scanned in an apparatus similar to that described by Loughman and Martin (1957). This apparatus was used by the courtesy of the I.C.I.A.N.Z. Central Research Laboratories, Melbourne.

EXPERIMENTAL RESULTS

The effect of varying the boron content of the medium on the growth of flax roots. Boron was added to the nutrient medium as boric acid before autoclaving to give concentrations ranging from 0 to 100 p.p.m. B. The final length of the roots after 7 days' incubation at 27° C. in media of various boron concentrations is given in Fig. 2. Two other experiments gave similar results.

The smallest concentration of boron for optimum growth lay between 0·01

and 0·05 p.p.m. B. This is similar to that found for flax seedlings grown for 3 weeks in nutrient culture (Neales, 1959).

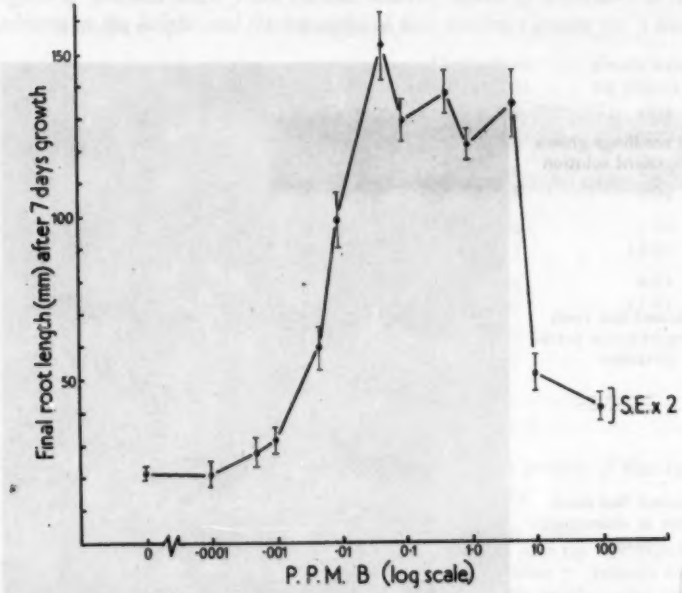


FIG. 2. The effect of B concentration on the growth in length of 10 mm. root tip explants cultured in a sterile medium. Vertical lines with the mean growth values level with their midpoints have vertical extents = 2 × standard error of the mean values.

At the end of each 7-day experiment the laterals on each root were counted. The boron-deficient roots typically were without laterals, whilst the maximum number formed on a +B root was twenty. The effect of boron supply on the mean number of lateral roots formed in 7 days from the growth of each 10 mm. root tip is indicated in Table IV. This effect of boron on lateral root growth is consistent with the view that the major effect of this element is on meristematic growth. Boric acid at concentrations above 5 p.p.m. B were toxic, and restricted growth in length and also lateral root development.

TABLE IV

The effect of varying boron concentrations on the growth of excised flax roots

p.p.m. B in medium.	Final root length after 7 days (mm.).	Mean number of laterals per root.
100	40·5±3·6	0
1·0	121·5±4·6	10
0·1	129·3±6·4	12
0·01	98·3±8·6	7
0·001	31·1±4·0	0
0·0001	20·3±3·8	0
0	21·4±1·5	0

It was observed from the daily inspection of the cultures that the effect of deficiency in the boron supply was to inhibit growth at a time after the beginning of the experiment dependent on the initial amount of boron added to the medium. Thus roots grown in cultures lacking boron ceased to grow

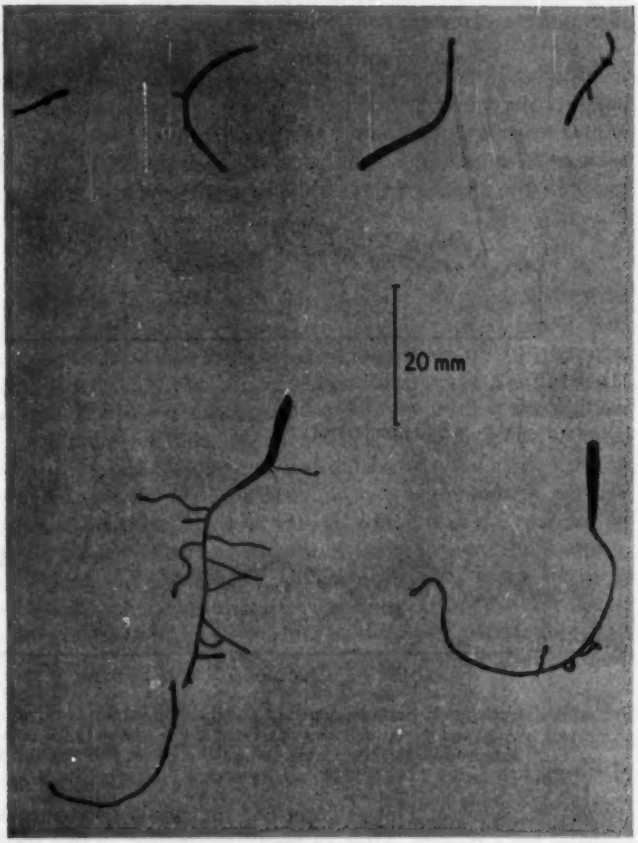


FIG. 3. Autoradiograph of flax roots grown for 3 days in 0·1 or 0 p.p.m. B, and subsequently placed in a solution containing C^{14} sucrose for 12 hours.

after approximately 48 hours, whilst the growth of those in a medium containing 0·005 p.p.m. B continued for 96 hours. Roots whose growth was stopped by lack of boron on day 2 were not able to recover when transferred to a +B medium on day 7. This is different to the behaviour of B-deficient flax seedlings grown in nutrient cultures, on whose roots new laterals always appear 48–72 hours after adding boron to the medium.

The entry of ^{14}C *sucrose into flax roots grown in sterile culture.* Two samples, each of 10 roots grown separately for 3 days in media containing 0·1 and 0 p.p.m. B respectively, were washed in sterile water. They were then added to a 0·5 per cent. boron-free sucrose solution to which had been added 0·11 mg.

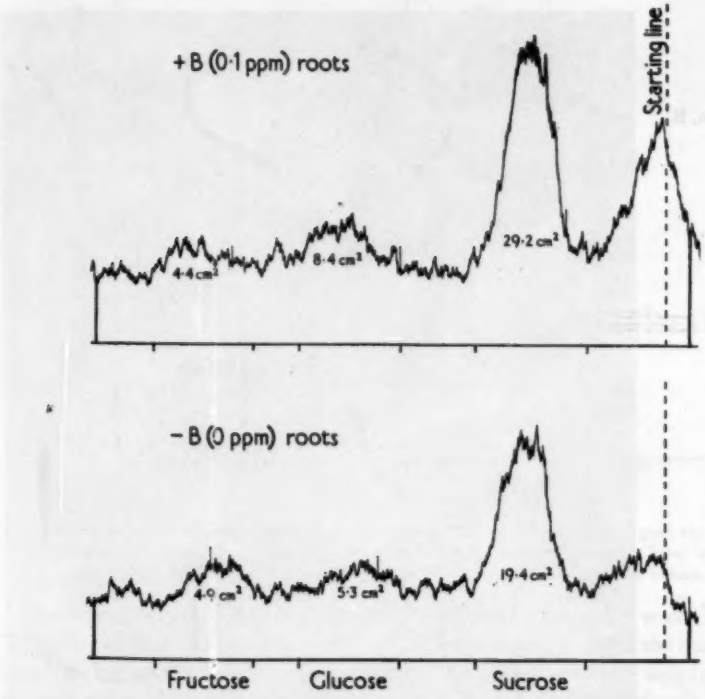


FIG. 4. Radioactivity on chromatograms of an ethanolic extract (equivalent to 5 mg. fresh weight) of normal and boron-deficient flax roots after incubating for 12 hours in a 0·5 per cent. sucrose solution containing 10 μc C^{14} sucrose. See text and Figure 3.

(10 μc) ^{14}C sucrose. This solution had previously been autoclaved at 15 lb. pressure for 1 minute. After 12 hours' incubation in this medium at 27° C. the roots were removed and separated into 'boron-deficient' and 'normal' roots. Each group was briefly dipped in distilled water and then washed five times in distilled water on a filter-paper in a Buchner funnel, each change of water being held in the funnel for 10 minutes before its removal by suction. Some of the roots were then transferred to hot 80 per cent. ethanol and stored, others were dried and autoradiographed.

The autoradiograph (Fig. 3) showed that there was no obvious difference in the radioactivity of normal and boron-deficient roots.

Aliquots of the ethanolic extracts of the normal and boron-deficient roots,

each equivalent to 5 mg. fresh weight, were applied to No. 3 Whatman paper and examined chromatographically, as already described.

The location of the radioactivity on the chromatogram (Fig. 4) indicates the presence of ^{14}C sucrose, glucose, and fructose. The quantities of these radioactive sugars on the chromatogram are proportional to the area described by the chart pen (above the background level) at Rf values corresponding to each sugar. These areas were measured on the original chart with a planimeter and are given in Fig. 4.

The results indicate the presence, in roots of both types, of twice as much ^{14}C sucrose as ^{14}C glucose plus ^{14}C fructose. The boron-deficient roots, whose growth had ceased, contained approximately 33 per cent. less sucrose than the rapidly growing (Fig. 3) plus-boron roots. An identical uptake of ^{14}C sucrose by boron-deficient and normal roots is unlikely in view of their very different morphology.

DISCUSSION

The boron requirement of excised roots. In previous investigations on the nutritional requirements of excised roots grown in sterile culture, precautions were not taken to eliminate boron contamination from boro-silicate glassware and glass-distilled water. All the following workers used pyrex-distilled water and pyrex glassware. Robbins and Schmidt (1938) reported that the addition of a 0·1 p.p.m. solution of B and Zn improved growth in White's (1934) medium. White (1938) found that the omission of boron caused a mean reduction of 24 per cent. in each of three consecutive weekly growth periods. Glasstone (1947) and Hannay and Street (1954) found that the omission of boron did not significantly affect the growth of tomato roots. Heller (1953, reported by Gautheret, 1955) recorded that the absence of boron for three weekly passages killed a culture of carrot-root phloem. These variable results can be attributed to contamination from the boro-silicate glassware, which would add an unknown and variable quantity of boron to the culture medium used (Table I).

The results presented in this paper demonstrate a boron requirement of excised flax roots which is satisfied by a concentration of 0·05 p.p.m. B. This is similar to the optimum requirement for intact flax seedlings grown in nutrient culture (Neales, 1959). Thus, in respect of boron nutrition at least, the contention of Glasstone (1947) that the mineral nutrition of excised roots is less complex than that of the whole plant is not valid. To date, the majority of investigations on the nutritional requirements of excised roots have been undertaken using tomato roots. However, it seems reasonable to suppose that the very severe effect of boron deficiency on excised flax roots reported in this paper could be reproduced in excised tomato roots, provided that adequate precautions in technique were adopted.

The inhibition, by boron deficiency, of the growth of flax roots is similar to the inhibition of the radicles of germinating beans (Whittington, 1957), who suggested that the absence of boron affected cell division. In reproducing

these results (Neales, unpublished), it was found that the sugar concentration in the tips of bean roots whose growth was restricted by lack of boron was high, and similar to that found in the tips of the rapidly growing 'plus boron' roots.

If the main function of boron in the intact plant was concerned in sugar translocation (Gauch and Dugger, 1953, 1954), then it might be expected that the boron requirement of excised flax roots, floating in a 4 per cent. sucrose solution, might be lower than that of the intact plant. The fact that this is not so supports the contention of Skok (1957, 1958) that the apparent influence of boron on sugar distribution and translocation is a secondary effect.

The uptake of sucrose by excised flax roots. The production of boron deficiency in flax roots, floating in a medium containing 4 per cent. sucrose, indicated a function of boron in growth unrelated to sugar translocation. There remained, however, the alternative hypothesis (Gauch and Dugger, 1954) that cell membranes of boron-deficient plants were impermeable to sugars. For this reason the uptake of ^{14}C sucrose by normal and boron-deficient flax roots was studied.

Roots, the growth of which had been inhibited by lack of boron, absorbed ^{14}C sucrose from a 0·5 per cent. sucrose solution (Fig. 4). Rapidly growing roots cultured in a medium containing 0·1 p.p.m. B absorbed 33 per cent. more than the deficient roots. The washing procedure adopted was such that the sucrose taken into the free space of the root tissues was removed before their extraction and analysis (Table V).

TABLE V

The removal, by successive washings for 10 minutes in 25 mls. H_2O, *of sugars from flax roots after incubation for 12 hours in a 0·5% sucrose solution*

Washing number	Time min.	Sucrose removed, μ gm./gm. f.w. of roots	%	Glucose plus fructose removed, μ gm./gm. f.w. of roots	%
1	0–10	3,433	80·0	5,450	85·1
2	10–20	608	14·1	550	8·6
3	20–30	183	4·3	317	4·9
4	30–40	57	1·3	38	0·6
5	40–50	9	0·2	50	0·8

The proponderence of ^{14}C sucrose over ^{14}C glucose and ^{14}C fructose in the roots (Fig. 4) indicates that either sucrose was absorbed as such (Porter and May, 1955; Weatherley, 1953) without prior hydrolysis; or that, if hydrolysis of sucrose did take place extracellularly (Harley and Smith, 1956; Burström, 1957, 1958), then there was rapid synthesis of ^{14}C sucrose in the cells of the roots, as has been reported by Putman and Hassid (1954) in leaf disks. Ferguson *et al.* (1958) have recently demonstrated with excised tomato roots that the full growth-promoting activity of sucrose is not reproduced by glucose and fructose, either separately or in a mixture, which indicates that sucrose is absorbed by cells without hydrolysis.

The uptake of ^{14}C sucrose by flax roots, whose growth is inhibited by lack of boron, indicates that this inhibitory effect is not due to the cells of boron-deficient flax roots being impermeable to sugars.

It therefore seems impossible to explain the effects of boron on the growth of excised flax roots in a sterile medium either in terms of an effect of boron on sugar translocation or on the permeability of cell membranes to sugar. This postulated role of boron in the movement of sugars in plants (Gauch and Dugger, 1953, 1954) has been criticized previously by Whittington (1957). Skok (1957, 1958), Nelson and Gorham (1957), and Neales (1959).

The function of boron in plant metabolism. Despite much experimental work (reviewed by Gauch and Dugger, 1954; and Skok, 1958) no unequivocal evidence has been produced indicating the basic reaction (or reactions) in the plant which are dependent upon the presence of boron.

These experiments with flax roots failed to give direct evidence of the part played by boron in their metabolism. The indirect evidence of the very severe inhibition of flax root growth in media lacking boron supported the conclusions that the processes of cell division (Whittington, 1957) and also cell maturation (Skok, 1958) are those primarily affected by lack of boron. There are also indications (Dugger, Humphreys, and Calhoun, 1957) that phosphorylation reactions are affected by boron nutrition.

ACKNOWLEDGEMENTS

The technical assistance of Mr. W. F. Buck is acknowledged. I am grateful to Professor J. S. Turner and Dr. K. S. Rowan for criticizing the manuscript, and also to Dr. M. J. Canny, of I.C.I.A.N.Z., Central Research Laboratories, Melbourne, for his generous help in the scanning of chromatograms for radioactivity.

LITERATURE CITED

Böeseken, J. (1949). The use of boric acid for the determination of the configuration of carbohydrates. *Adv. Carbohydrate Chem.* **4**, 189.

Bonner, J., and Devirian, P. S. (1939). Growth factor requirements of four species of isolated roots. *Amer. J. Bot.* **26**, 661.

Burström, H. (1957). Root surface development, sucrose inversion and free space. *Physiol. Plantarum.* **10**, 747.

—— (1958). Influence of plasmolysis and inhibitors on the sucrose inversion in wheat. Ibid. **11**, 771.

Dugger, W. M. Jr., Humphreys, T. E., and Calhoun, B. (1957). The influence of boron on starch phosphorylase and its significance in translocation of sugars in plants. *Plant Physiol.* **32**, 364.

Ferguson, J. D., Street, H. E., and David, S. B. (1958). The carbohydrate nutrition of tomato roots. V. The promotion and inhibition of excised root growth by various sugars and sugar alcohols. *Ann. Bot.* N.S. **22**, 513.

Gauch, H. G., and Dugger, W. M. Jr. (1953). The role of boron in the translocation of sucrose. *Plant Physiol.* **28**, 457.

—— —— (1954). The physiological action of boron in higher plants: a review and interpretation. *Bull. Maryland Agric. Exp. Sta.* A 80 (technical).

Gautheret, R. J. (1955). The nutrition of plant tissue cultures. *Ann. Rev. Plant Physiol.* **6**, 433.

Glasstone, V. F. C. (1947). Inorganic micronutrients in tomato root tissue culture. *Amer. J. Bot.* **34**, 218.

HANNAY, J. W., and STREET, H. E. (1954). Studies on the growth of excised roots. III. The molybdenum and manganese requirement of excised tomato roots. *New Phytol.* **53,** 68.

HARLEY, J. L., and SMITH, D. C. (1956). Sugar absorbtion and surface carbohydrase activity of *Peltigera polydactyla* (Neck.) Hoffm. *Ann. Bot.* N.S. **20,** 513.

HEWITT, E. J. (1952). Sand and water culture methods used in the study of plant nutrition. *Tech. Commun. Bur. Hort.*, E. Malling, No. 22.

KRAUSS, R. W. (1958). Physiology of fresh water algae. *Ann. Rev. Plant Physiol.* **9,** 207.

LANGRIDGE, J. (1957). The aseptic culture of *Arabidopsis thaliana* (L.) Heynh. *Australian. J. Biol. Sci.* **10,** 243.

LOUGHMAN, B. C., and MARTIN, R. P. (1957). Methods and equipment for the study of the incorporation of phosphorus by intact barley plants in experiments of short duration. *J. Exp. Bot.* **8,** 280.

NEALES, T. F. (1959). The effect of boron nutrition on the sugars, soluble in 80% ethanol, in flax seedlings. *Nature*, Lond., **183,** 483.

NELSON, C. D., and GORHAM, P. R. (1957). Uptake and translocation of C^{14}—labelled sugars applied to the primary leaves of soyabean seedlings. *Canadian. J. Bot.* **35,** 339.

PORTER, H. K., and MAY, L. H. (1955). Metabolism of radioactive sugars by tobacco leaf discs. *J. Exp. Bot.* **6,** 43.

PUTMAN, E. W., and HASSID, W. Z. (1954). Sugar transformations in leaves of *Canna indica*. I. Synthesis and inversion of sucrose. *J. Biol. Chem.* **207,** 885.

ROBBINS, W. J., and SCHMIDT, M. B. (1938). Growth of excised roots of tomato. *Bot. Gaz.* **99,** 671.

SISLER, E. C., DUGGER, W. M. Jr., and GAUCH, H. G. (1956). The role of boron in the translocation of organic compounds in plants. *Plant Physiol.* **31,** 11.

SKOK, J. (1957). The substitution of complexing substances for boron in plant growth. Ibid. **32,** 308.

—— (1958). *The role of boron in the plant cell. Trace elements.* Academic Press Inc. New York.

STREET, H. E. (1957). Excised root culture. *Biol. Rev.* **32,** 117.

WEATHERLEY, P. E. (1953). On the uptake and hydrolysis of sucrose by leaf tissues. *New Phytol.* **52,** 76.

WHITE, P. R. (1934). Potentially unlimited growth of excised tomato root tips in a liquid medium. *Plant Physiol.* **9,** 585.

—— (1938). Accessory salts in the nutrition of excised tomato roots. Ibid. **13,** 391.

WHITE, L. M., and SECOR, G. E. (1953). Occurrence of two similar homologous series of oligosaccharides in wheat flour and wheat. *Arch. Biochem. Biophys.* **43,** 60.

WHITTINGTON, W. J. (1957). The role of boron in plant growth. I. The effect on general growth, seed production and cytological behaviour. *J. Exp. Bot.* **8,** 353.

On the Distribution of Gentisic Acid in Green Plants

L. A. GRIFFITHS

Regional Research Centre, Imperial College of Tropical Agriculture, Trinidad, B.W.I.

Received 8 January 1959

SUMMARY

Gentisic acid has been shown to be one of the most commonly occurring aromatic acids of green plants. The systematic distribution of the compound is described and its possible relationship to lignification is discussed. Methods for its separation from other phenolic constituents of plants and for the identification of the compound are described.

INTRODUCTION

In a preliminary report from this laboratory the widespread occurrence of gentisic acid in Angiosperms was noted (Griffiths, 1958 *a*). In the present paper the distribution of gentisic acid in a large number of families of tropical plants is described; the distribution of gentisic acid and other phenolic acids throughout the vegetative and reproductive tissues of *Theobroma cacao* has been earlier reported (Griffiths, 1958 *b*).

Although there are many reports in the earlier literature of the sporadic distribution of phenolic acids in plant tissues (Bonner, 1950), it is largely the result of recent chromatographic studies that the ubiquity of certain of these compounds has been appreciated. Evidence of the widespread occurrence of ferulic, sinapic, caffeic, *p*-coumaric acid, and ellagic acid has been presented by Bate-Smith (1954, 1956 *a* and *b*); the distribution of the chlorogenic acid isomers has been described by Sondheimer (1958) and evidence of the widespread occurrence of the *p*-coumarylquinic acids, first isolated by Cartwright *et al.* (1955), has recently been obtained by Griffiths (1958 *c*). Current interest in these compounds centres on their relationship to the biogenesis of flavonoids (Bogorad, 1958), to lignin biosynthesis (Brown and Neish, 1955, 1956), and their role in the mechanism of disease resistance to plant pathogens (Johnson and Schaal (1957); Kuć *et al.* (1956)).

In the earlier studies on the occurrence of gentisic acid in plant tissues (Griffiths 1958 *a*), the presence of gentisic acid in *Theobroma cacao* L. was established by the use of five different solvent systems. Of these solvents, benzene-acetic acid-water (60:70:30) was shown to be the most effective and was selected for the screening procedure employed in the current studies.

MATERIALS AND METHODS

Of the leaf material collected for this survey, only mature leaves were selected for analysis, as the content of phenolic acids was observed earlier to vary with the physiological age of the tissue (Griffiths, 1958 *b*).

Extraction and chromatography. 2 g. of fresh, finely shredded leaf tissue were digested with 10 ml. of 20% aqueous hydrochloric acid (v/v) for 20 min. on a boiling water bath. After cooling and filtering, the filtrate was extracted with 2 ml. of diethylether and 0·1 ml. of this applied to a sheet of Whatman No. 4 chromatography paper, which was formed into a cylinder and developed with benzene-acetic acid-water (60:70:30) by ascending chromatography. The original procedure (Griffiths, 1957) was modified only in that the chromatograms were routinely run at 20° C. instead of 15° C. employed in the earlier experiments. The higher temperature was observed to result in an appreciable rise in the Rf values recorded. The dried chromatograms were then examined in ultra-violet light and the gentisic acid spots (Rf 0·28) located by their blue fluorescence. All extracts were run against an authentic standard of gentisic acid (Delta Chemical Works, Inc.)

The amount of gentisic acid present in a hydrolysate may be gauged by the size and intensity of the fluorescent spots on the chromatogram. By visual comparison with standard amounts of gentisic acid applied to the chromatogram, gentisic acid was estimated to be present in amounts ranging from 0 to *c.* 100 μg./g. fresh leaf material.

A further criterion for the identification of gentisic acid on chromatograms is provided by the changes in fluorescence of the compound in the presence of ammonia. On exposure to fumes of ammonia solution (sp. gr. 0·910) a greening of the gentisic acid spot occurs. When the chromatogram is, on the other hand, sprayed with aqueous ammonia solution (sp. gr. 0·910) the original blue fluorescence changes to a bright yellow. This response to spraying with strong ammonia solutions is not given by any of the more common phenolic constituents of leaf hydrolysates, although *o*-coumaric acid, which is of relatively rare occurrence, does produce a similar U.V. coloration on spraying. *o*-Coumaric acid is easily distinguished from gentisic acid, however, by virtue of the high Rf of the former substance (0·76 at 20° C.).

The use of *p*-nitraniline (Swain, 1953) is of little value in the detection of gentisic acid as the coloration produced is extremely faint. In view, moreover, of the complexity of the hydrolysed extracts, which usually contain much phenolic material of low Rf, the $FeCl_3/K_3Fe(CN)_6$ reagent of Barton *et al.* (1952) is insufficiently specific for routine screening, although in view of its sensitivity it was of value in the detection of gentisic acid in less complex extracts.

RESULTS

The presence of gentisic acid was shown in a high percentage of plants examined. In Table I, a list is given of the species in which positive identifications of gentisic acid were made. It is important to note that gentisic acid, although detected in representatives of each of the families listed, is not necessarily present in all genera belonging to the group, the amount of gentisic acid being found to vary between genera and species. Whilst the degree of lignification is a factor of importance with regard to this variation there are

undoubtedly other factors responsible for this effect. The physiological age of the material has been shown to be important in *Theobroma cacao* in determining the leaf content of phenolic acids (Griffiths, 1958 *b*).

TABLE I

List of Families and representative Genera in which positive identifications of gentisic acid were made

Acanthaceae, *Barleria cristata*; Amaranthaceae, *Amaranthus spinosus, Alternanthera ficoidea*; Anacardiaceae, *Mangifera indica*; Annonaceae, *Annona muricata*; Apocynaceae, *Kopsia* sp., *Thevetia peruviana*; Araliaceae, *Didymopanax morototoni*; Asclepiadaceae, *Sarcostemma clausum*; Bignoniaceae, *Crescentia cujete, Tabebuia pentaphylla*; Bixaceae, *Cochlospermum vitifolium*; Bombacaceae, *Ochroma lagopus*; Boraginaceae, *Cordia sebestina*; Burseraceae, *Protium guianense*; Cactaceae, *Rhipsalis cassytha, Opuntia* sp.; Capparidaceae, *Cleome ciliata*; Caricaceae, *Carica papaya*; Caryophyllaceae, *Dianthus* sp.; Casuarinaceae, *Casuarina equisetifolia*; Combretaceae, *Terminalia catappa*; Compositae, *Cosmos bipinnatus, Chrysanthemum coronarium*; Coniferae, *Cupressus sempervirens*; Cucurbitaceae, *Cucurbita maxima, Momordica charantia*; Dilleniaceae, *Dillenia indica*; Ebenaceae, *Diospyros discolor*; Erythroxylaceae, *Erythroxylum novogranatense*; Euphorbiaceae, *Acalypha hispida*; Flacourtiaceae, *Hydnocarpus anthelminthica*; Gramineae, *Bracharia mutica, Pennisetum purpureum*; Guttiferae, *Mesua ferrea*; Lauraceae, *Cinnamomum camphora*; Lecythidaceae, *Couroupita guianensis*; Leguminosae, Papilionatae, *Dipteryx odorata, Andira inermis, Cajanus indicus, Arachis hypogea, Crotalaria anagyroides, Samanea saman*; Caesalpinioideae, *Caesalpinia pulcherrima*; Mimosoideae, *Mimosa pudica*; Loganiaceae, *Buddleia davidii*; Lythraceae, *Lagerstroemia indica*; Magnoliaceae, *Michelia champaca*; Malpighiaceae, *Galphimia glauca*; Malvaceae, *Hibiscus esculentus, H. rosasinensis, Malvaviscus grandiflorus*; Marantaceae, *Calathea altissima*; Marcgraviaceae, *Marcgravia hartii*; Moraceae, *Castilloa elastica*; Myristicaceae, *Myristica fragrans*; Myrsinaceae, *Ardisia acuminata*; Myrtaceae, *Eucalyptus deglupta, Psidium guajava*; Nyctaginaceae, *Bougainvillea glabra*; Ochnaceae, *Ochna mossambicensis*; Oleaceae, *Jasminum sambac*; Oxalidaceae, *Averrhoa carambola*; Palmae, *Cocos nucifera, Oreodoxa regia*; Pandanaceae, *Pandanus* sp.; Passifloraceae, *Passiflora auriculata*; Plumbaginaceae, *Plumbago* rubra; Polygonaceae, *Triplaris* sp.; Portulacaceae, *Portulaca pilosa*; Rhizophoraceae, *Rhizophora mangle*; Rosaceae, *Rosa* sp.; Rubiaceae, *Portlandia grandiflora, Coffea excelsa, Morinda citrifolia*; Rutaceae, *Citrus aurantium, Aegle marmelos*; Sapindaceae, *Litchi chinensis*; Sapotaceae, *Chrysophyllum cainito, Achras zapota*; Saxifragaceae, *Hydrangea* sp.; Scrophulariaceae, *Scoparia dulcis*; Simarubaceae, *Quassia amara*; Solanaceae, *Lycopersicum esculentum*; Sterculiaceae, *Theobroma cacao, T. grandiflora, T. microcarpa, Herrania balaensis*; Theaceae, *Camellia* sp.; Tiliaceae, *Apeiba* sp.; Turneraceae, *Turnera* sp. Umbelliferae, *Apium graveolens*; Urticaceae, *Fleurva aestuans*; Vitaceae, *Leea coccinea*; Zygophyllaceae, *Guaiacum officinale*.

Although neutral methanolic extracts of leaf material were examined, no free gentisic acid was detected and it appeared that preliminary hydrolysis was essential for the release of the acid from the complex in which it occurs. A methanol-soluble, fluorescent, gentisic acid-containing complex was subsequently isolated from leaves of *Theobroma cacao* by chromatography in butanol-acetic acid-water.

Methanolic extracts prepared by the standard technique (Griffiths, 1958 *b*) were examined by band-chromatography in butanol-acetic acid-water (40:10:50). After development and drying, the chromatogram was cut into ten strips of equal width, parallel to the base-line, and the strips heated in 20 per cent. aq. HCl at 100° C. for 30 minutes. A second chromatogram prepared similarly was screened for U.V. fluorescent compounds and the fluorescent bands cut out and submitted to hydrolysis. It was shown that a gentisic acid-yielding

compound was present on the chromatograms at a position corresponding with an Rf 0·25 and that the compound coincided in position with a band showing a deep blue fluorescence. The absence of any other gentisic acid-containing complex in the methanolic extract was indicated by the complete absence of gentisic acid in hydrolysates obtained from the remaining areas of the chromatogram. The possibility that gentisic acid might also occur in the leaf in the form of a methanol-insoluble complex was examined. Leaf material was homogenized in methanol in a Microblender and after filtration the residue was exhaustively washed by repeated resuspension in methanol. When this material was submitted to hydrolysis under the standard conditions no gentisic acid could be detected in the residue. It is evident therefore that all of the gentisic acid detected in the leaf of *T. cacao* originates from the methanol-soluble complex of Rf 0·25.

No correlation was observed between the distribution patterns of gentisic acid and those of the other fluorescent phenolic acids of the leaf hydrolysates.

DISCUSSION

It will be observed (Table I) that gentisic acid has been detected in both herbaceous plants and trees. It is, however, important to note that the compound was of more frequent occurrence and tended to be present in larger amounts in many of the families included by Hutchinson (1946) in the Lignosae than was observed in the Herbaceae or the Monocotylae. It is also notable that the Angiosperm families, in which gentisic acid could not be detected (Table II), belong to either the Herbaceae or the Monocotylae. Gentisic acid was with few exceptions detected in the leaves of all trees examined in this survey. It was, however, absent from the two Cycads, *Cycas*

TABLE II

List of Families in which the gentisic acid content of leaves was found to be invariably low or absent

Angiosperms:

Aristolochiaceae (H)
Balsaminaceae (H), Begoniaceae
Bromeliaceae (H), Campanulaceae (H)
Cannaceae (M)
Commelinaceae (M)
Convolvulaceae (H), Crassulaceae (H)
Cyclanthaceae (M)
Gesneriaceae (H)
Labiatae (H), Liliaceae (M)
Musaceae (M), Nymphaeaceae (H)
Orchidaceae (M), Piperaceae (H)
Polemoniaceae
Pontederiaceae (M)
Zingiberaceae (M)

Other groups in which gentisic acid was absent:

Cycadaceae, Filices.

M, Monocotylae; H, Herbaceae

revoluta and *Zamia* sp. and the genera *Musa* and *Ravenala* of the Musaceae. It is of interest also to note that gentisic acid was absent from all species of fern examined in this survey.

If gentisic acid is concerned in lignification, it is necessary to envisage translocation from the leaf to the cambium. It is of interest to note, therefore, that the presence of gentisic acid has been shown in the green stem and sapwood of *T. cacao* (Griffiths, 1958 *b*).

The association of gentisic acid with a woody habit in plants suggests that the substance is concerned in lignification or in some associated process. Degradative studies on lignin have not, however, yielded any fragment showing a similar type of hydroxylation nor has any substituted cinnamic acid showing 2:5 hydroxylation been isolated from leaf material. It is evident, therefore, that if gentisic acid is a precursor of lignin, modification of the hydroxylation pattern must occur at an early stage in biosynthesis, prior to the formation of the phenyl-propane carbon skeleton. Although the incorporation of ^{14}C labelled shikimic acid and phenyl-propanes into the lignin molecule has been clearly demonstrated (Brown and Neish 1955 *a* and *b*), little is known of the pathway linking the former intermediates, although the formation of phenyl-propane (C_6—C_3) units by the addition of a C_2 to a C_6—C_1 unit has been suggested (Brown and Neish, 1955 *b*).

The aromatic C_6—C_1 compounds investigated by Brown and Neish (1955 *b*) were found to be ineffective precursors of lignin and further work on the nature of the intermediates at this stage of biosynthesis is clearly desirable. In view of the widespread distribution of gentisic acid in woody plants the possibility that the compound is a precursor of the phenyl-propane skeleton should be explored, although the presence of an ortho-hydroxy group remains to be explained and suggests that the compound may be the product of a side reaction of the aromatization pathway.

LITERATURE CITED

Barton, G. M., Evans, R. S., and Gardner, J. A. F. (1952). Paper chromatography of phenolic substances. *Nature*, **170**, 249.

Bate-Smith, E. C. (1954). Ferulic sinapic and related acids in leaves. *Chem. and Ind.* 1457.

—— (1956 *a*). The commoner phenolic constituents of plants and their systematic distribution. *Sci. Proc. Roy. Dubl. Soc.* **27**, 165.

—— (1956 *b*). Chromatography and systematic distribution of ellagic acid. *Chem. and Ind.* 32.

Bogorad, L. (1958). The biogenesis of flavonoids. *Ann. Rev. Plant Physiol.* 417.

Bonner, J. (1950). *Plant Biochemistry*. New York, Academic Press Inc.

Brown, S. A., and Neish, A. C. (1955 *a*). Shikimic acid as a precursor in lignin biosynthesis. *Nature*, **175**, 688.

—— —— (1955 *b*). Studies of lignin biosynthesis using isotopic carbon. Formation from aromatic monomers. *Canad. J. Biochem. Physiol.* **33**, 948.

—— —— (1956). Studies of lignin biosynthesis using isotopic carbon. Comparative studies on different plant species. *Canad. J. Biochem. Physiol.* **34**, 769.

Cartwright, R. A., Roberts, E. A. H., Flood, A. E., and Williams, A. H. (1955). The suspected presence of *p*-coumaryl-quinic acids in tea, apple and pear. *Chem. and Ind.* 1062.

GRIFFITHS, L. A. (1957). Separation and identification of aromatic acids in plant tissues by paper chromatography. *Nature*, **180**, 286, 1957.

—— (1958 *a*). Occurrence of gentisic acid in plant tissues. Ibid., **182**, 733.

—— (1958 *b*). Phenolic acids and flavonoids of *Theobroma cacao* L. Separation and identification by paper chromatography. *Biochem. J.* **70**, 120.

—— (1958 *c*). Unpublished observations.

HUTCHINSON, J. (1946). *A Botanist in Southern Africa*. London, P. R. Gawthorn.

JOHNSON, G., and SCHAAL, L. A. (1945). Chlorogenic acid and other ortho-dihydric phenols in resistant and susceptible potato tubers. *Phytopathology*, **47**, 253.

KUĆ, J., HENZE, R. E., ULLSTRUP, A. L., and QUACKENBUSH, F. (1956). Chlorogenic and caffeic acids as fungistatic agents produced by potatoes in response to inoculation with *Helminthosporium carbonium*. *J. Amer. Chem. Soc.* **78**, 312.

SONDHEIMER, E. (1958). On the distribution of caffeic acid and chlorogenic isomers in plants. *Arch. Biochem. Biophys.* **74**, 131.

SWAIN, T. (1953). Identification of coumarins and related compounds by filter paper chromatography. *Biochem. J.* **53**, 200.

Micro-Nutrient Deficiency and Transpiration in *Tropaeolum majus* L.

KARL H. SCHÜTTE

Department of Botany, University of Cape Town

SUMMARY

The transpiration of *Tropaeolum* plants, grown in water culture, on full nutrient solutions and on solutions deficient in one of the following, i.e. boron, copper, manganese, and zinc, was studied. The transpiration rate of the control plants grown on the full nutrient solution was significantly higher than that of the deficient ones. The more acute the deficiency, the greater was the disparity.

Under the conditions of the experiment, the control plants were smaller in size, having fewer and smaller leaves than the deficient ones, but the foliage was much greener and healthier in appearance.

INTRODUCTION

THE influence of micro-nutrient status upon the transpiration of plants has hardly been investigated, although reference to their importance in other aspects of water relations are more frequently made. Boas (1942) stresses the fact that applications of boric acid, zinc sulphate, and magnesium sulphate have a marked beneficial influence upon the drought resistance of red clover. Deficiencies have been noted to increase susceptibility to wilting in several species (Willis and Piland, 1937; Schütte, 1957).

Boas (1942), Dorfmüller (1941), Bieble (1942), and Schmied (1953) conducted experiments in the field, and found that the addition of boron compounds to the soil increased the transpiration of the plants so treated. But these were all additive experiments in which no clear symptoms of deficiency are described. It is possible that the addition of certain salts may have a stimulating influence upon the transpiration rate of normal plants. Also, their transpiration measurements were based upon the water losses of excised leaves. These measurements are difficult to interpret, so that the results of these experiments must be accepted with caution, as they may be more complex than they at first appear.

Baker, Gauch, and Dugger (1956) investigated the influence of boron upon various aspects of water relations in snap-beans, sunflowers, and tomatoes grown in sand culture and showed that the rate of transpiration of boron deficient leaves was appreciably less than that of plants receiving boron. Leaf's (1953) experiments with controlled medium gave the same results with *Nicotiana tobacum* var.

Except for the work of Schmied, who showed that the addition of boron, copper, manganese, and zinc compounds to the soil tended to increase the rate of transpiration in most of the plants that he investigated, there appears to be no information on the influence of micro-nutrients other than boron on transpiration.

Journ. of Experimental Botany, Vol 10, No. 30, pp. 443-47, Oct. 1959.

The present work deals with the influence of deficiencies of boron, copper, manganese, and zinc upon the transpiration rate of *Tropaeolum majus* L. This plant was chosen because it grows rapidly, is well suited to water culture, and it is possible to measure its leaf area accurately and rapidly. This facilitates the accurate measurement of its transpiration rate. To overcome the criticisms that can be levelled against additive experiments, a subtractive method was used, whereby only one nutrient at a time was removed from the full nutrient solution, thus assuring a deficiency of the absent element.

METHODS AND MATERIALS

Seeds of *Tropaeolum majus* L. var. Orange Gem were germinated in trays of acid washed sand, which were watered regularly with de-ionized water. When they were two inches high, they were transplanted to 300 ml. jars of nutrient solution.

The seedlings were wrapped in cotton-wool, for support in the neck of the jar. This plug of cotton-wool allowed air to reach the roots, yet cut down evaporation very effectively. The jars were wrapped in thick brown paper to keep light off the nutrient solution and the roots. The solution was renewed regularly, and the jars were filled to two-thirds of their volume, so that the roots had adequate aeration, because as transpiration occurred, the level of the liquid dropped rapidly. Thus root development was good.

Robbins's (1946) nutrient solution was used. The control plants had all essential nutrients present. The deficient solutions consisted of Robbins's solution lacking one salt only. Solutions deficient in boron (—B), copper (—Cu), manganese (—Mn), and zinc (—Zn) were used.

Each treatment consisted of 12 pots with one plant per pot. They were placed in a randomized manner in an unheated glass-house in order to eliminate any differences in behaviour due to differences in the environment. The glass-house was warm and dry. Temperatures of around 30° C. and humidities of 45–55 per cent. R.H. were frequently experienced for several hours per day.

Transpiration was measured by the loss in weight of the jar and its contents. Increases in weight of the plants were ignored. Investigation showed that the amount of water lost through the cotton-wool plug by evaporation was so small that it could be ignored.

When the jars were weighed to measure the loss of water, the leaf area was also measured. This was done by using callipers to measure the largest and smallest diameters of the leaf, and could be done without damaging the plants in any way. As the leaf of the nasturtium is so regular, the following formula for leaf area gave accurate results within 5 per cent. of the true area measured by exact outline of the leaf: area $= \pi\left(\frac{D1+D2}{4}\right)^2 = 0{\cdot}196(D1+D2)^2$.

As all the plants were grown in the same changing environment, and all treatments were comparable, the transpiration rates were comparable. As

conditions in the glass-house varied considerably, daily transpiration rates calculated from the available information could not be accurate.

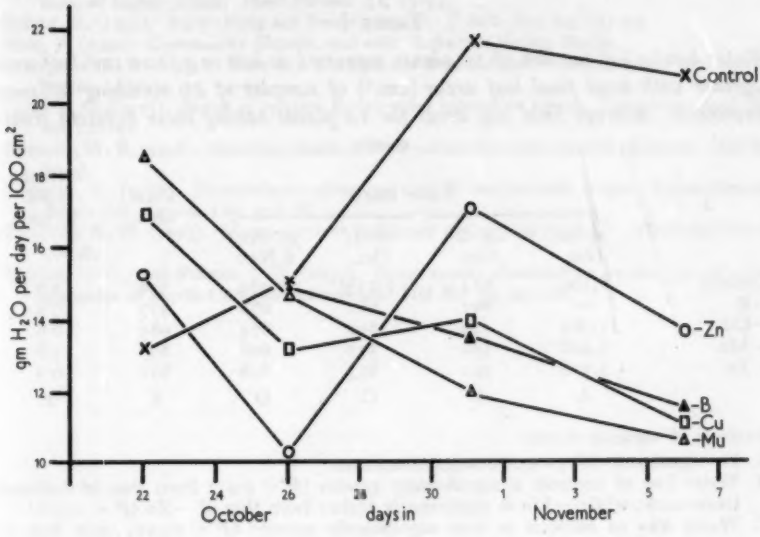


FIG. 1

RESULTS

Under the warm, dry conditions under which the plants were grown, the control plants were much smaller than those grown in deficient solutions. Both the size of their leaves and their number were significantly smaller than those of the deficient plants. On the other hand, their pigmentation was more intense, and they did not exhibit any signs of chlorosis. Their foliage, while small, was very healthy in appearance.

While all the deficient plants were larger than the controls, they did not present such a healthy appearance. The leaves were a much lighter shade of green, but acute chlorosis only occurred on a few leaves. Some leaves wilted and were removed from the plants. In general, the foliage was more fragile in appearance. Some details of the vegetative nature of the plants and their transpiration rates are shown in the table. The transpiration rates of the young plants in the different treatments did not differ significantly, cf. column A. This presumably is due to the fact that the mineral reserves of the seeds had not been exhausted and so, as yet, no appreciable deficiencies had set in. But as the plants grew, deficiencies became apparent and the transpiration rate of the deficient plants became progressively slower than that of the controls as the deficiencies became more acute.

As well as differing from the controls, the various treatments differed among themselves. The —Mn and —Zn treatments differed significantly

from each other, but in this experiment the other treatments did not differ significantly from each other.

TABLE I

Water loss (g.) of batches of 10 plants expressed as loss in g./100 cm² leaf area together with total final leaf areas (cm.²) of samples of 10 receiving different treatments. Average final leaf areas for 12 plants having these different treatments

	Water loss				Total area	Average area p. leaf
	14–22 Oct.	22–26 Oct.	26–31 Oct.	31 Oct.–6 Nov.		
Control	1,104	614	1,213	1,379	330	3·3
−B	—	601	682	678	977	7·1
−Cu	1,363	499	692	674	985	6·6
−Mn	1,428	556	572	608	895	5·8
−Zn	1,318	391	813	816	871	6·3
	A	B	C	D	E	F

Analysis of variance shows:

A. No significant difference between treatments.
B. Water loss of controls is significantly greater ($P < 0.01$) from that of deficient treatments; while −Mn is significantly higher from that of −Zn ($P < 0.01$).
C. Water loss of controls is very significantly greater ($P < 0.001$) than that of deficient plants; −Mn is significantly lower ($P < 0.05$) than −Zn.
D. Water loss of controls is very significantly greater ($P < 0.001$) than that of deficient plants; −Mn is significantly lower ($P < 0.01$) than −Zn.
E. Very significant ($P < 0.001$) difference between the area of the control and that of the deficient plants which did not differ very significantly.
F. Average leaf areas of control plants are very significantly smaller ($P < 0.001$) than those of the deficient plants. Leaves of the −Mn plants are significantly smaller ($P < 0.02$) than those of the −B ones.

DISCUSSION

This investigation substantiates the claims of previous investigators that micro-nutrient deficiencies are associated with a decrease in the transpiration rate of plants. These results indicate that while the deficiencies are associated with, and probably the cause of, a decrease in the rate of transpiration, this decrease is not the same in each deficiency. −Zn appears to be quite distinct in its response to −B, −Mn, and −Cu, which may indicate that different stages in the process are influenced.

ACKNOWLEDGEMENTS

I wish to acknowledge with gratitude the assistance of Mr. J. Darroch in statistical matters; and of Mr. E. Rösemann for technical assistance. Part of this work was subsidized by a C.S.I.R. grant, which made possible the assistance of Mr. Rösemann, and for which I should like to express my thanks.

LITERATURE CITED

BAKER, J. E., GAUCH, H. G., and DUGGER, W. M. (1956). Effects of boron on the water relations of higher plants. *Plant Physiol.* **31**, 89–94.

BIEBLE, R. (1942). Borwirkung auf Pisum sativum. *J. wiss. Bot.* **90**, 731–49.

BOAS, F. (1942). *Dynamische Botanik*, 2nd edit. Lehmans Verlag. Berlin.

DORFMÜLLER, W. (1941). Über den Einfluss des Bors auf den Wasserhaushalt der Leguminosen. *Planta*, **32**, 51–65.

LEAF, L. G. (1953). Boron in relation to the water balance in plants. *Proc. Iowa Acad. Sci.* **60**, 176–91.

ROBBINS, W. R. (1946). Growing plants in sand culture for experimental purposes. *Soil Sci.* **62**, 3.

SCHMIED, E. (1953). Spurenelementdüngung und Wasserhaushalt einiger Kulturpflanzen. *Öster. Bot. Zeitsch.* **100**, 552–78.

SCHÜTTE, K. H. (1957). Micro-nutrient deficiency and wilting in radish. *Plant and Soil*, **9**, 186–8.

WILLIS, L. G., and PILAND J. R. (1937). Some recent observations on the use of minor elements in North Carolina agriculture. *Soil Sci.* **44**, 251–61.

Observations on the Internal Structure of the Spermatozoid of *Dictyota*

I. MANTON

(*Botany Department, Leeds University*)

SUMMARY

The spermatozoid of *Dictyota* is shown to be structurally more like a vegetative zoospore than was the case in *Fucus*. The mitochondria, golgi, overall shape of the nucleus, nuclear membrane, fat bodies, and miscellaneous vesicles are as in a zoospore; there is, however, a vestigial chromatophore without an eyespot and the ciliary apparatus is specialized. In spite of the single flagellum there are two basal bodies inside the cell, one of which is apparently vestigial, ending blindly in the cytoplasm; both have a similar internal structure with nine fibres in the wall. A fibrous 'root', possibly homologous with the 'proboscis' of *Fucus*, arises near the outer side of the functional basal body; it is a band of about eight fibres passing through the cell without touching the nucleus but closely pressed to the inner faces of at least two mitochondria before ending in an unknown manner at the cell surface. The internal structure of the flagellum is normal but the fact that the median strand is marked by a row of spines has been used to demonstrate in a conclusive way the facts of bilateral symmetry in the whole organ; *Dictyota* agrees exactly with *Fucus* and a previous error based on incomplete information has been corrected. The process of unwinding of the flagellum from the surface of the body after liberation from the antheridium is described and used to illustrate some unexpected properties of the surface membrane.

INTRODUCTION

THE spermatozoid of *Dictyota* is unique in several respects. The alga is one of the most striking examples known, and the first to be discovered (Lloyd Williams, 1897, 1898, 1905), of periodic liberation of gametes in precise relation to certain spring tides. The spermatozoid itself is the best example of a uniflagellate cell (Lloyd Williams, 1897, 1905) in the Phaeophyceae proper, the motile cells of which are characteristically biflagellate with two laterally attached flagella, one projecting forward and the other backward. Finally, the flagellum, while projecting forward and provided with the two rows of lateral hairs characteristic of anterior flagella in most heterokont types, is in this case provided with a third row of externally visible appendages (Manton, Clarke, and Greenwood, 1953). This unique feature consists of a row of about a dozen blunt spines situated on what appears to be the median line between the two rows of hairs and all attached to one only of the internal fibres. Since the flagellar axis possesses the normal internal structure of 9+2 fibres, it is difficult to avoid the conclusion that the fibre so marked is the median one.

This last fact is the reason for the special interest of this material since it provides a more precise method of determining the plane of bilateral symmetry in the flagellum as a whole and of correlating external with internal

Journ. of Experimental Botany, Vol. 10, No. 30, pp. 448-61, Oct. 1959.

structure than can be obtained from any other known plant. It would therefore undoubtedly have been sectioned sooner, as part of an extended programme on the structure and morphology of cilia and flagella in plants generally, had it been more readily accessible. The periodic liberation, limited to at most 6–10 days in the year, is, however, a considerable obstacle which has required repeated attempts in several successive years before it could be surmounted. For this reason alone it would have been undesirable to limit observation to flagellar structure. It is impossible to section flagella in isolation from the cells which bear them, and the cells contain many features of considerable interest both to algologists and to students of fine structure. This paper is therefore a general account of the microanatomy of the spermatozoid of *Dictyota* in which the structure of flagella is included, but only as one among several topics.

MATERIAL AND METHODS

Attempts have been made to fix material of *Dictyota* almost annually since 1954, with varying success, the most satisfactory years having been 1955 and 1958, in each case utilizing one or other of the September spring-tide periods. Since it is known from the pioneer work of Lloyd Williams (1905) that the precise pattern of relation to the spring tides varies on different parts of the coast according to the time of day at which low water occurs at the peak of the springs, it is essential to have intimate knowledge of a particular locality before collecting can be carried out on the right day. For this reason, after one unsuccessful attempt at Plymouth, attention has been concentrated on Lloyd Williams's own collecting ground in the Menai Straits (North Wales) for which maximum natural liberation occurs at low water on the 3rd or 5th tide after the lowest of the spring tides in September. Since artificial liberation can be obtained 24 hours earlier if plants are gathered and stored in a cool place out of water, it is possible to collect fertile material on the occasion of the maximum spring ebb, and sometimes on the day before this, at either of the two September periods and obtain liberation next day.

It should, however, be noted that in addition to the tidal periodicity *Dictyota* seems to possess a diurnal rhythm which prevents effective swimming in the afternoon,[1] even on the right day, though the same thalli if kept for 2 days out of water will swim effectively on the morning of the second day though not on the afternoon of the first. Second-day swimming is less vigorous than first-day swimming.

Collecting has been done either by dredging from a boat at mid-afternoon before low water on the east side of rocks between the two bridges that connect Anglesey with the mainland (this region is that known as 'The Swillies' in Lloyd Williams's paper). This is an almost unfailing source of good material

[1] According to Lloyd Williams (1905) non-motile gametes liberated in the afternoon can be stimulated to a short period of activity by standing the vessel in direct sunlight. We were unaware of this possibility at the time of our own experiments but we have no reason to doubt its correctness and this device could obviously be very helpful since otherwise it is impossible to distinguish between living cells capable of being fixed with reasonable chance of success and dead ones for which this is entirely impossible.

if dredging is carried out on the right day. Equally good material was obtained on one occasion by wading at the north end of Trearddur Bay on Anglesey where, on a sandy bottom, large boulders carry an excellent growth of permanently submerged *Dictyota* which can be reached at a peak spring tide.

The best method of storage is to wrap selected male thalli in a bundle tightly in clean paper or a clean cloth to absorb excess water and then to put the bundle so wrapped into a plastic bag or glass jar which should be kept out of the sun and left over night in some cool place such as a cellar or outdoors in the shade; chilling by refrigeration should be avoided.

To obtain liberation, selected male thalli should be rinsed in clean seawater and spread out in petri-dishes in just enough filtered seawater to cover them. They should be left undisturbed for a few minutes (½ hour for the late September tide in 1958 when the experiment was started at 9 a.m., probably rather less for the comparable day and time at the earlier September tide). During this period the dishes should be examined under low power of a microscope from time to time. Once liberation starts it generally proceeds rapidly and on a scale sufficient to cause a visible cloudiness in the water. In a successful swim a drop of this water placed on a slide with a pipette will contain an intensely active swarm of motile gametes which may be dense enough to be squirted directly into the fixative or which may need to be further concentrated by centrifugation; they cannot be concentrated by light. Motility is short lived and is generally over within 20 minutes from the start of liberation.

The fixative used throughout has been the standard osmium tetroxide buffered with acetate veronal. In 1955, a year with an exceptionally fine warm summer and which yielded, perhaps in consequence, some very good observations (see Pl. VIII) the pH used was 8·2 and the best results were from centrifuged material for which a 1 per cent. osmium tetroxide solution was directly poured on to the centrifugate which was left undisturbed till dehydration was complete, the tube meanwhile being stored in ice. In subsequent years pH 7 has been preferred and the best results from the 1958 fixations were obtained by squirting the natural material without centrifugation directly into an equal volume of 2 per cent. osmium tetroxide at this pH in a tube chilled in ice. The effective strength of fixative in both cases was thus 1 per cent.

Fixation times of ½ hour and 1 hour have both been used satisfactorily, after which the material is spun down, the fixative poured off, and the pellet rinsed with the briefest possible contact with buffer solution to remove excess of fixative which is immediately replaced by 30 per cent. alcohol kept cool with ice. Thereafter dehydration and embedding procedures are standard.

All the successful fixings were carried out in the laboratory of the Marine Biological Station at Menai Bridge, using reagents brought from Leeds. All fixations were made into conical centrifuge tubes of 3·5 c.c. capacity and centrifugation was carried out on a Baird and Tatlock micro-angle centrifuge (55°) with the speed reduced by means of a rheostat to slightly less than

maximum to minimize the risk of tube fracture. The embedding temperature was 48° C.

The blocks were mostly cut in Leeds using a Porter-Blum Sorval microtome and a glass knife. Some of the figures on Pl. VIII were, however, obtained from three preparations cut in New York in 1956 from the best block of the 1955 fixation but left unexamined from lack of time in America and lack of a suitable microscope in Leeds. These three preparations which had been mounted on carbon films on large-sized punched grids were cut down to the smaller size required by the Siemens microscope in 1958, with surprisingly little damage either from this process or the 2 years' storage.

Owing to the length of time during which the investigation has been on hand a great many different microscopes have been used. The work was begun in 1954 using the low resolution Philips microscope in the Leeds Botany Department. In 1956 this was supplemented by access to a new Philips microscope carrying the 25A pole piece with astigmator which was made available to me for 1 week at the Philips factory at Eindhoven, and from which Figs. 4, 27*a* and *b*, 28*a–d* are reproduced. Further observations were made on the RCA microscope at the Rockefeller Institute, New York, during a 2 weeks' visit at Christmas 1956, from which Figs. 19, 26, and 30 are reproduced. Finally the main body of observations have been carried out on the new Siemens microscope installed in 1958 in the Botany Department at Leeds by means of a grant from the Rockefeller Foundation; the material for this, except for the three preparations mentioned in the previous paragraph, was fixed in September 1958.

OBSERVATIONS WITH THE LIGHT MICROSCOPE

The description given by Lloyd Williams in 1905 is so excellent and in such close agreement with most of our observations that it is profitable to quote it verbatim. His drawing of spermatozoids killed with iodine and examined with an oil immersion objective is also reproduced (Fig. 3, Pl. I) since it faithfully represents what can be seen by a most skilful observer using the light microscope. His description (loc. cit., p. 190) is as follows:

> Taking first the antherozoid as it appears in the living condition, we find that it is pear-shaped while active, but spherical when it comes to rest. At the broader, posterior end there is a globular colourless part which with chromatin stains takes on an intense coloration, showing it to be the nucleus. The pointed end is occupied by granular protoplasm in which there is always a very minute red eyespot, sometimes two as in the lowest example in the figure. Instead of being situated close to the attachment of the cilium as is usual in other antherozoids and in zoospores, the eyespot here is always at a distance from it, frequently at the pointed anterior end. The cilium is not terminal as was stated in the '97 paper, but lateral as in other Phaeophyceae; this fact can be clearly seen only while the antherozoid is in motion. The cilium is attached at the posterior edge of the cytoplasmic cone, near its junction with the nucleus. Very often there seems to be a slight thickening at the very base, but on this point it is not easy to speak with certainty.

The only additional observation that can usefully be added to this description is that the living spermatozoids in bulk are so nearly colourless that even when centrifuged the resulting pellet is only faintly tinged with pink. The spermatozoids seem also to be insensitive to the direction of incident light and cannot be concentrated by it (cf. p. 450). These facts are pertinent to a true appraisal of the nature of the body referred to as an eyespot and it is instructive to quote an earlier description by the same author to show that he also was aware of something anomalous about it (Lloyd Williams, 1897, p. 548):

With regard to the spherical bodies above-mentioned, it may be further added that in some cases a very faint tinge of yellow is observable in them. This suggests the possibility of their representing the 'chromatic spot' characteristic of the antherozoids of Fucus. Though smaller and much less obvious than the latter, they are quite easily recognisable in the antheridia of Dictyota, Taonia and Haliseris. In motile antherozoids they often seem to project beyond the periphery of the head as also do the eyespots in the antherozoids of Fucus. The study of fixed material however makes it rather doubtful that these granules represent eyespots. In the first place they are not always present, and even when they occur, their position has no definite relation to the points of insertion of the cilia.

As will be shown below, this genuine dilemma is easily resolved with the electron microscope in the sense that a vestigial chromatophore is almost certainly present in the place assigned to the 'spherical body' but, unlike *Fucus*, this body does not contain an eyespot though it is doubtless the seat of the faint vestigial pigmentation found. We may therefore accept entirely the various observations contained in both these descriptive paragraphs quoted from Lloyd Williams whilst emending his interpretation on this one point.

DESCRIPTION OF PLATES I–IV

Plate I

Fig. 1*a* and *b*. Two adjacent longitudinal sections of a spermatozoid of *Dictyota* passing through the flagellum and nucleus (*NN*), selected to show the irregular shape of the clear area within the nucleus. S632.12 and S632.13. ×15,000.

Fig. 2. A similar cell showing the position of the ciliary base relative to the nucleus (*NN*); two fat bodies and some vesicles in the cytoplasm. H2413. ×25,000.

Fig. 3. Spermatozoids as seen under oil immersion with the light microscope, reproduced from Lloyd Williams 1898.

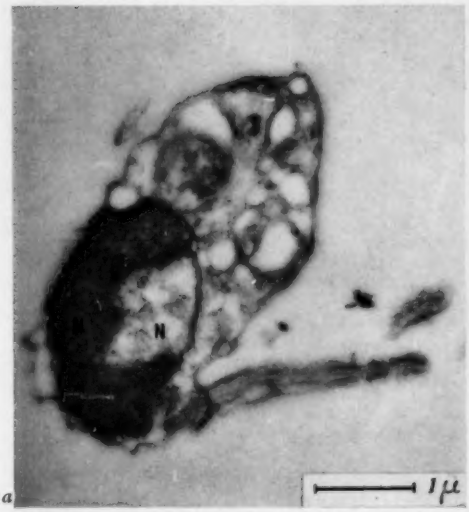
Plate II

Fig. 4. LS through the two basal bodies, the upper one carrying the functional flagellum, the lower ending blindly; a piece of the nucleus (*N*), and a mitochondrion (*m*). E394.18. ×*c.* 50,000.

Fig. 5. TS showing the two basal bodies, also the nucleus (*N*), a mitochondrion (*m*) and the putative vestigial plastid (*p*). S632.14. ×*c.* 20,000.

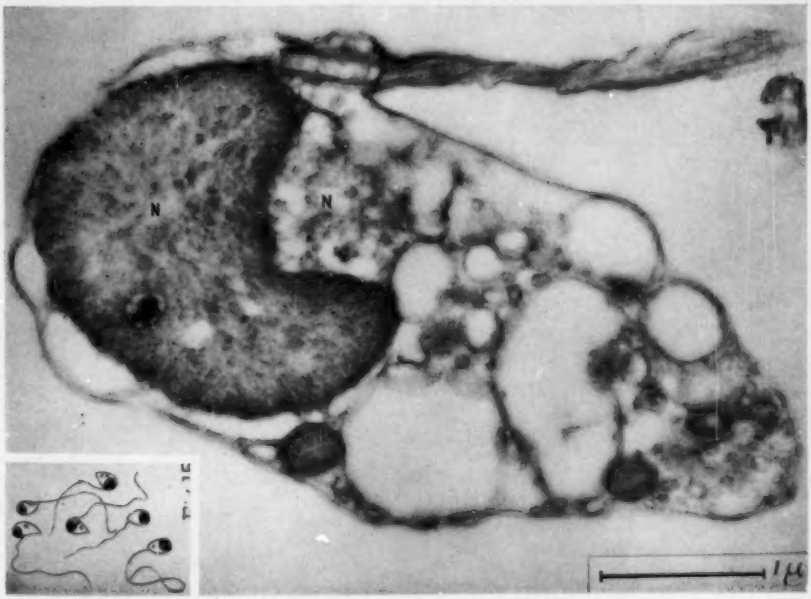
Figs. 6*a* and *b*. Two sections through a pair of basal bodies, the upper one the functional base and the lower the vestigial base; the nucleus (*N*) and the flagellar root in TS (*r*) M2609. ×30,000 (Fig. 6*a*); M2540. ×60,000 (Fig. 6*b*).

Fig. 7. LS of the functional base in relation to the nucleus (*NNN*) cut in a plane at right angles to that of Fig. 4, showing the double nuclear membrane (*nm*) H2470. ×50,000.



1a

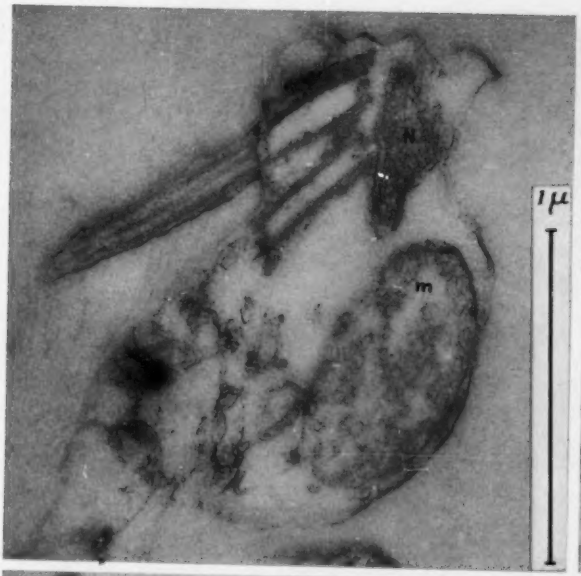
1b



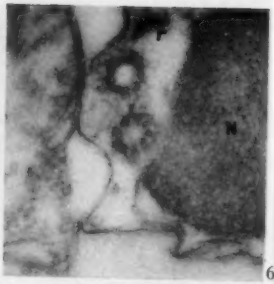
2

3

I. MANTON—PLATE I



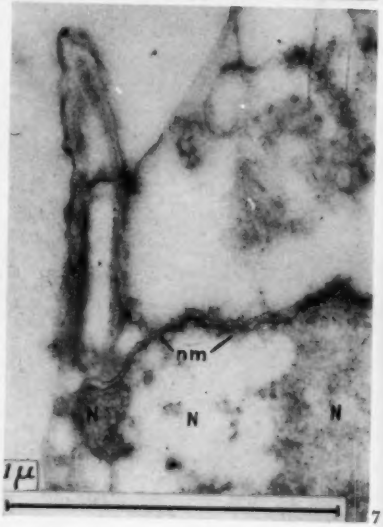
4



6a

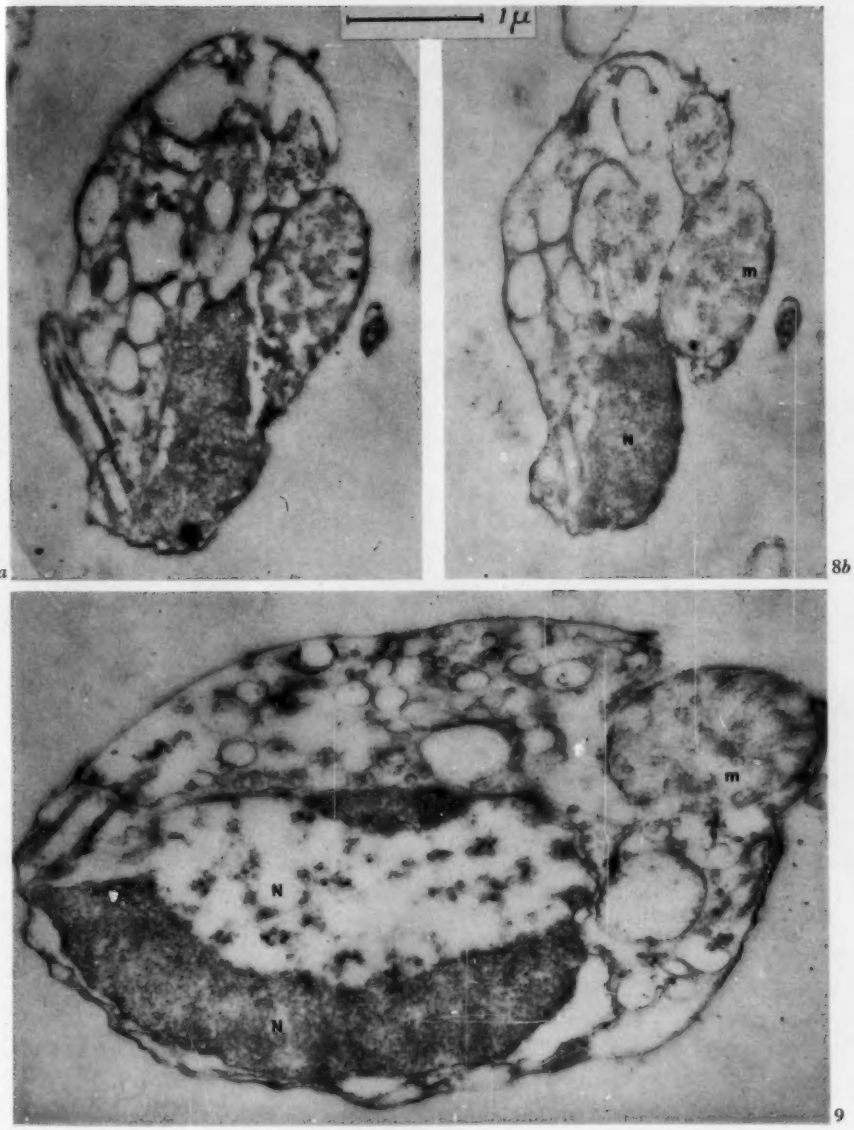
6b

5



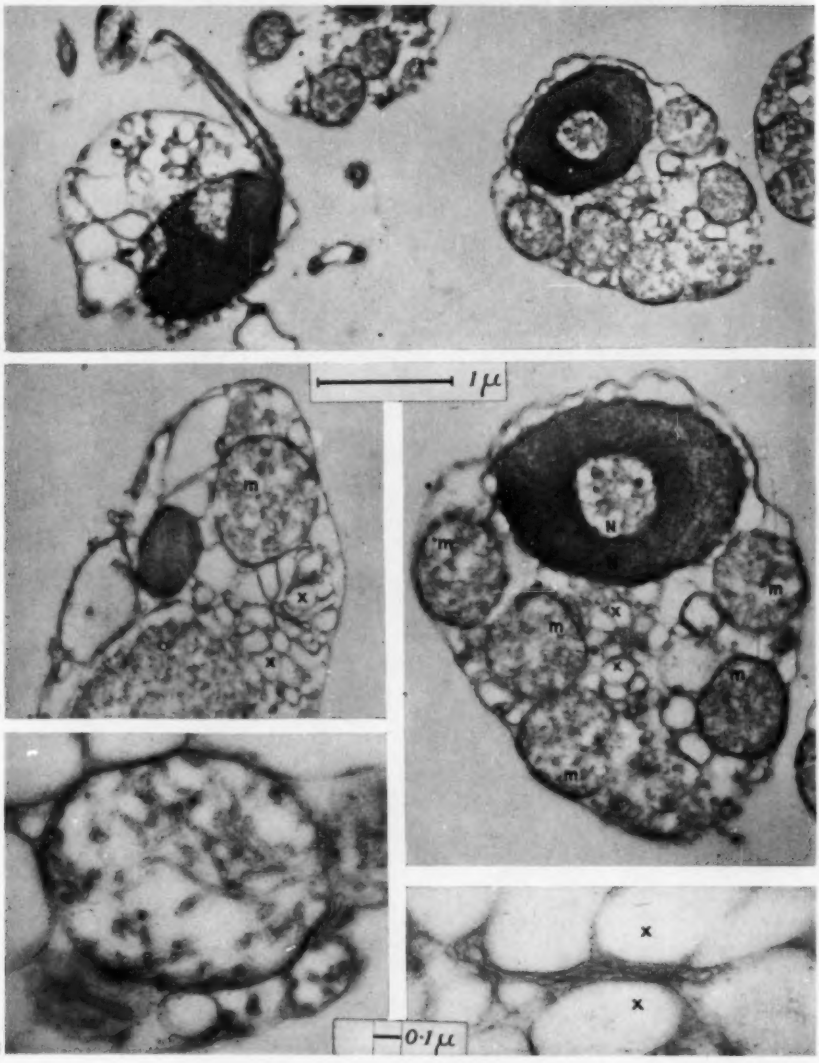
7

I. MANTON—PLATE II



8a 8b 9

I. MANTON—PLATE III



I. MANTON—PLATE IV

NEW OBSERVATIONS WITH THE ELECTRON MICROSCOPE

The external morphology of the spermatozoids of *Dictyota* will be found fully illustrated in Manton, Clarke, and Greenwood (1953) and reference to that paper is necessary for the full details (see, however, the general statement on p. 448). When sections are used the most easily interpretable plane is that in which the flagellum and cell-body are both traversed longitudinally as on Pl. I. Comparison with Lloyd Williams's drawing (Fig. 3) shows a gratifyingly close agreement in the position and shape of the various parts visible with the light microscope which include the posterior attachment of the single flagellum and the posteriorly placed nucleus.

The nucleus. This agreement is important since without it the position and shape of the nucleus might give grounds for uncertainty. The nucleus has in fact a normal rounded shape but it is not of uniform opacity throughout. There is a very translucent area of irregular shape which at first might be interpreted as cytoplasm occupying a nuclear depression. This area is, however, never separated from the denser part of the nucleus by any structure resembling a nuclear membrane but is manifestly covered by a membrane in common with the rest of the nucleus (see especially Figs. 1*a* and *b*). There seems therefore little doubt that it is indeed part of and not outside the nucleus. It gives rise to very curious appearances in different planes of section (e.g. Pls. I, IV, and elsewhere) but where doubt might arise the initial letter *N*, denoting nucleus, has been inserted in both light and dark parts of the nucleus in many of the illustrations.

The nuclear membrane. This has been demonstrated more clearly here than in *Fucus* for which fixation was too imperfect for it to be detectable (Manton and Clarke, 1956). At low powers the nuclear membrane is well seen as a line of demarcation between nucleus and cytoplasm (Figs. 1, 2*b*, &c.) but that it is also a double membrane comparable in a general way to that known for other organisms is clear from more highly magnified micrographs such as Figs. 7 and 9.

PLATE III

FIG. 8*a* and *b*. Two sections through a cell selected to show the median LS through each of the two basal bodies in a plane at right angles to that of Fig. 4: Fig. 8*a* the functional base, Fig. 8*b* the vestigial base. H2477 and H2484. ×20,000.

FIG. 9. A section through a better fixed specimen showing the relation of the vestigial base to the cell membranes, the nucleus (*N*), and the cell surface. H2485. Magnification uncertain, probably between 30,000 and 40,000.

PLATE IV

FIG. 10. A field with two cells cut in planes approximately at right angles, the right-hand cell shown larger in Fig. 12. H2417. ×11,000.

FIG. 11. Tip of a cell showing a fat body and a laterally placed golgi body (*xx*) at the surface between a mitochondrion (*m*) and the nucleus. H2484. ×20,000.

FIG. 12. The right-hand cell of Fig. 10 more highly magnified to show the nucleus (*NN*), 5 mitochondria (*m*), and golgi (*xx*); the section cut tangentially to the cell surface and at right angles to that of Fig. 11. H2416. ×20,000.

FIG. 13. A mitochondrion showing tubular internal villi. ×40,000.

FIG. 14. A golgi area showing the paired flattened lamellae between vesicles. H2501. ×40,000.

Mitochondria. These are relatively small and fairly numerous (e.g. Fig. 12), traces of up to six being often obtainable in a single section and this is unlikely to be the entire complement; structurally they are of the normal phaeophycean type with numerous, almost filiform, internal microvilli (Fig. 13, &c.). Their distribution, scattered through the front (cytoplasmic) part of the cell, resembles that of a zoospore (*Scytosiphon*) rather than the spermatozoid of *Fucus* which was remarkable (Manton and Clarke, 1956) for the small number, large size, and conspicuously fixed position of its mitochondria relative to the eyespot. This difference will be discussed again.

Golgi. This was not found in the *Fucus* spermatozoid but was clearly present in the *Scytosiphon* zoospore (Manton and Clarke, 1957) as a group of characteristic flattened vesicles near the attachment of the cilia. A similar group is present in *Dictyota* and is illustrated in Figs. 11, 12, and 14, Pl. IV (labelled *x x*), situated close to the cell surface (Fig. 11) immediately anterior to the nucleus (Figs. 11 and 12) and therefore necessarily near to the base of the flagellum. The fine structure with characteristic vesicles and flattened paired membranes is best seen in Fig. 14.

The flagellum. As explained in the introduction this organ is of particular interest in connexion with a special anatomical problem concerning cilia and flagella generally. This aspect of its structure is, however, by no means the first to strike the eye in viewing sections nor, from the point of view of algology, is it the most interesting. The anomaly of the single flagellum is the most conspicuous feature to receive an immediate descriptive explanation, when sections are examined in almost any plane other than those selected for Pls. I and IV. If attention is directed to Pls. II and III it is at once obvious that, single as the flagellum undoubtedly is when viewed externally, there are two basal bodies within the cell as in other brown algae, but in this case the second base ends blindly in the cytoplasm.

Fig. 4 is a good example of a section passing longitudinally through the two basal bodies one of which (uppermost in the figure) is carrying the flagellum, and the other, of comparable size and shape but without a flagellum, abuts on to the surface of the nucleus at its posterior end. Figs. 8*a* and *b* are two sections selected from a series cut at right angles to that of Fig. 4 and in which the two basal bodies are each displayed but in different sections. The second basal body (Fig. 8*a*) again ends blindly in the cytoplasm forwards and abuts obliquely on to the nuclear surface behind. A more highly magnified view of the second basal body in another case is contained in Fig. 9.

In transverse sections, e.g. Fig. 5, the two basal bodies are so much alike that at first it may be difficult to distinguish them. Both undoubtedly possess a wall composed of 9 strands (Figs. 6*a* and *b*). In addition, however, the functional base possesses a strap-shaped fibrous root (labelled *r* in Figs. 6*a* and *b*). This is described in greater detail below, but wherever it is visible it is always associated with the basal body actually carrying the flagellum which therefore, in the cell of Figs. 6*a* and *b*, is the upper one.

Pls. VI and VII give further information about the flagellar bases and the

related root. Pl. VI assembles various planes of section showing the root (*r* in most figures) in relation to other organs. It arises near the bottom end of the base of the functional flagellum (Fig. 25), on the side away from its functionless fellow. It runs beside the basal body for a short distance without fusing with it and then passes through the cell without touching the nucleus (Fig. 21), makes close contact with the inner faces of at least two mitochondria which can frequently be seen to be flattened against it at their contact faces (Figs. 21, 23, 24, 25) before it reaches the surface of the cell where it appears to end. The precise details of the ending cannot be traced and the internal structure of the root is hard to analyse since fixation is rarely good enough to give more than its position. For this purpose the 1955 alkaline fixation was the best though unfortunately the sections had become heavily contaminated in the various preliminary examinations with low-resolution microscopes before usable pictures could be obtained. Figs. 27*a* and *b* are from this material showing two different levels in one cell. Fig. 27*a* is the lower, showing the two bases clearly, with a mass of fibrous material outside the uppermost base from which the root arises. Fig. 27*b* is farther out; the second (lower) basal body has terminated blindly into a faint grey mark while the functional base shows the root (*r*) cut in TS and beginning to become apposed to an adjacent mitochondrion. In its contaminated condition this section cannot yield a picture sufficiently clear to be effective after half-tone reproduction, but in its original condition it justified the statement that the root is a band of parallel fibres of the order of 8 in number.

Another view of the functionless basal body ending blindly in the cytoplasm is contained in Fig. 30, Pl. VII which can also serve to introduce the coiled condition of the spermatozoid as it is liberated from the sporangium. Other views of the coiled spermatozoid are contained in Figs. 26 and 31, Pl. VII while Pl. V is arranged to illustrate the process of uncoiling, which has necessarily to be completed before swimming is possible.

In the coiled condition the whole length of the flagellum is wrapped round the cell several times and sections of the flagellar axis can be encountered, often repeatedly (see arrows in Figs. 15 and 26), always, however, beneath the body membrane and without any trace of separate membranes of their own (Fig. 19). The functionless basal body is in its usual position (Fig. 30); the functional base is, however, flexed inwards so that the flagellar axis at first lies against the nuclear surface before reaching the outside of the cell (Fig. 16).

Uncoiling seems to begin distally. Fig. 17 represents a partly uncoiled cell in an attitude which shows unmistakably that the flagellar membrane can be nothing other than a part of the body membrane which has been uplifted. Fig. 18 shows a cell with uncoiling almost though not quite complete in which the diaphragm delimiting the flagellum from its own base is clearly preformed within the cell but has not yet made contact with the surface (compare this with the vestigial base of Fig. 9, Pl. III).

Before uncoiling has occurred it is sometimes possible to detect the flagellar spines as projections below the body membrane on the cell surface. Some

examples are indicated by the letter *s* in Figs. 30 and 31*b*, and there is also a slight suggestion (Fig. 31*a*) that the flagellar hairs (Flimmer) may also project beyond the body membrane at an early stage before uncoiling has taken place. The evidence here is, however, not yet conclusive.

Spines on the fully expanded flagellum are shown in Pl. VIII, Fig. 32. They appear to be relatively opaque lumps beneath the flagellar membrane and the details of how they are attached to the fibre which bears them have not been ascertained. That they are nevertheless very firmly attached to one fibre only has been shown in considerable detail in a previous communication (Manton, Clarke, and Greenwood, 1953) and the position of this fibre relative to the rest of the flagellum can now be ascertained from transverse sections.

In studying flagellar symmetry by means of transverse sections in the region of a spine in *Dictyota* it is necessary to make careful allowance for distortion in cutting. The spine is slightly harder than the rest of the organ and when encountered obliquely by the knife is very liable to be pushed sideways. This is clearly demonstrated in Figs. 35*a* and *b* which are two immediately adjacent sections through a flagellum in the right region. Fig. 35*a* merely grazes the spine leaving the flagellum slightly flattened but undistorted. In Fig. 35*b* the spine has been directly hit and thereby pushed sideways; the plane of symmetry can nevertheless be reconstructed with certainty by collating both

DESCRIPTION OF PLATES V–VIII

Plate V

Fig. 15. A newly emerged coiled spermatozoid with the flagellum (arrows) still within the body membrane; three mitochondria and the vestigial plastid visible among vesicles in the cytoplasm. H2573. ×20,000.

Fig. 16. A coiled spermatozoid seen in another plane; arrows point to the flagellar base cut longitudinally and lying against the nucleus (*NN*). H2478. ×20,000.

Fig. 17. A cell partly uncoiled showing the body membrane lifting up to form the flagellar membrane. S636.17. ×20,000.

Fig. 18. An almost uncoiled cell showing the flagellar base nearly though not quite at the cell surface. H2479. ×20,000.

Fig. 19. Surface of a cell resembling that of Fig. 15 more highly magnified to show the absence of an individual membrane round the submerged flagellum; three sections of it and a fat body visible beneath the body membrane. R73D. ×*c.* 30,000.

Plate VI

Fig. 20. Oblique TS through the two basal bodies showing the 'root' attached to one of them and passing back to the cell surface after touching two mitochondria. H2591. ×20,000.

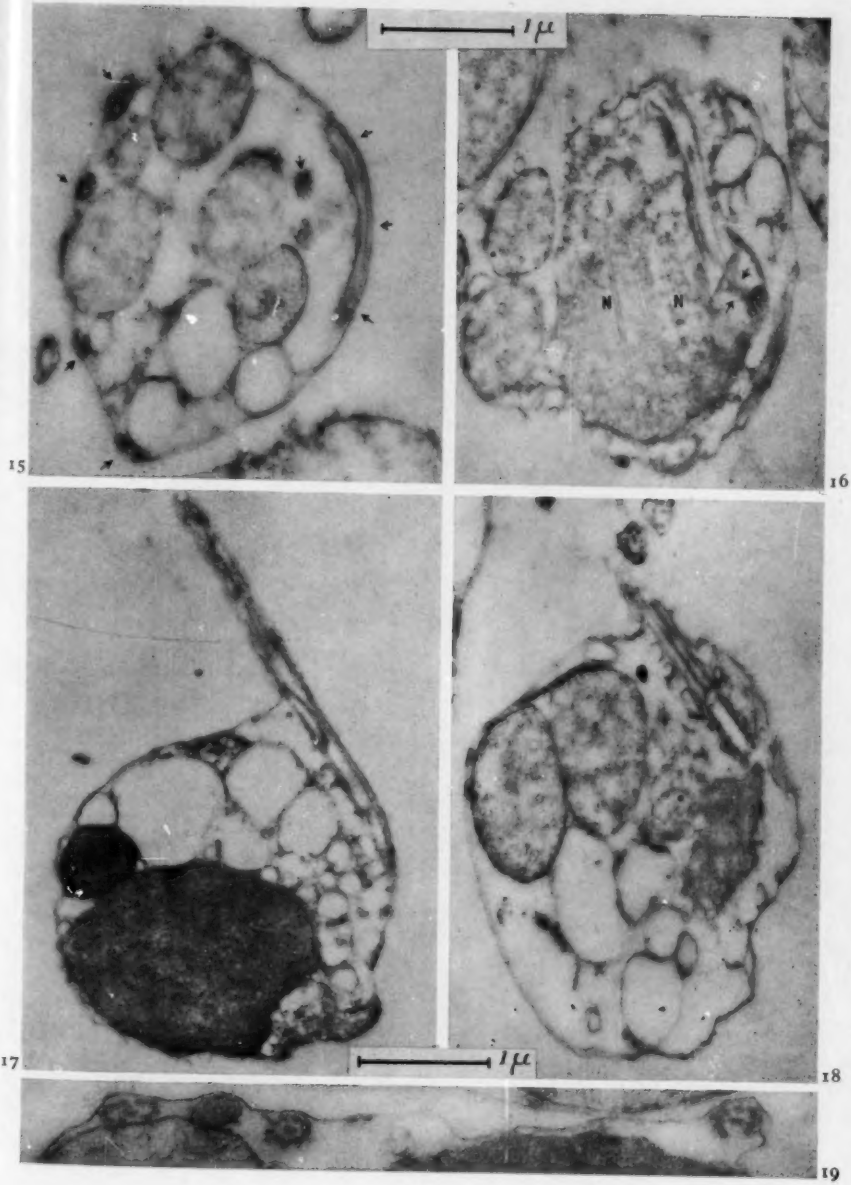
Fig. 21. Section showing the 'root' (*r*) passing back from the base of the functional flagellum towards but not touching the nucleus (*N*) and touching a mitochondrion (*m*) on its inner side which is flattened. H2518. ×20,000.

Fig. 22. Another view of the 'root', the tangentially grazed flagellum (arrows) belonging to the same cell visible on the left. S633.28. ×25,000.

Fig. 23. LS of a flagellum and its 'root' (*r*) passing over the flattened surface of a mitochondrion (*m*), the cell membrane (*bm*) very faint to the left near it. S643.28. ×20,000.

Fig. 24. An oblique section showing the 'root' (*r*) as a band, passing to the surface after touching two mitochondria. H2463. ×15,000.

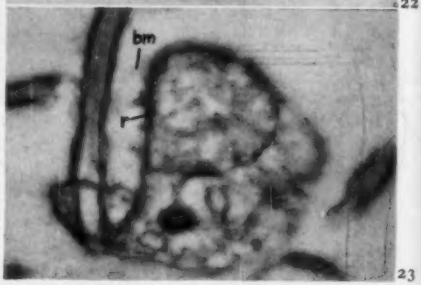
Fig. 25. LS through the flagellum and its 'root' (*r*) showing relation of this to a mitochondrion and the flagellar base. H2599. ×25,000.



I. MANTON—PLATE V

20

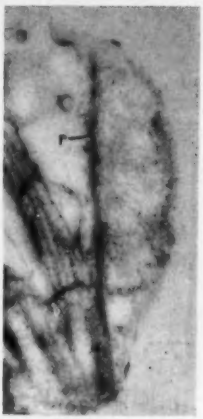
22



23

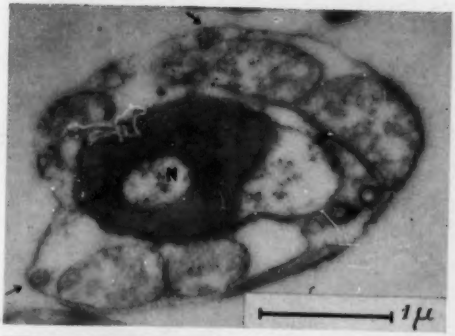
21

24

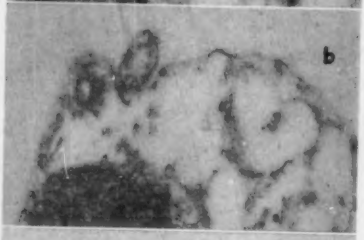


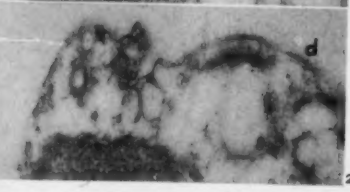
25

I. MANTON—PLATE VI

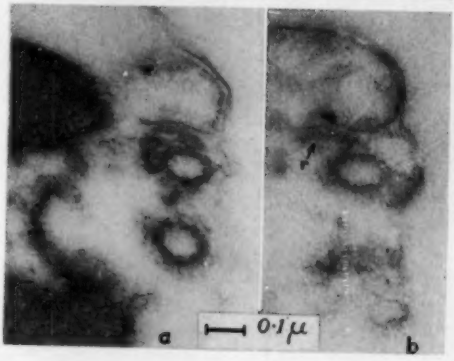


26

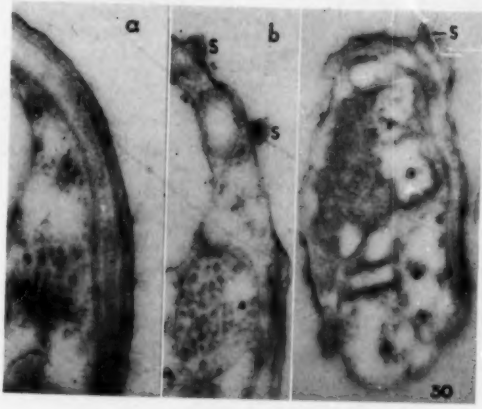




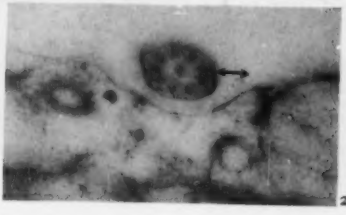
28



27

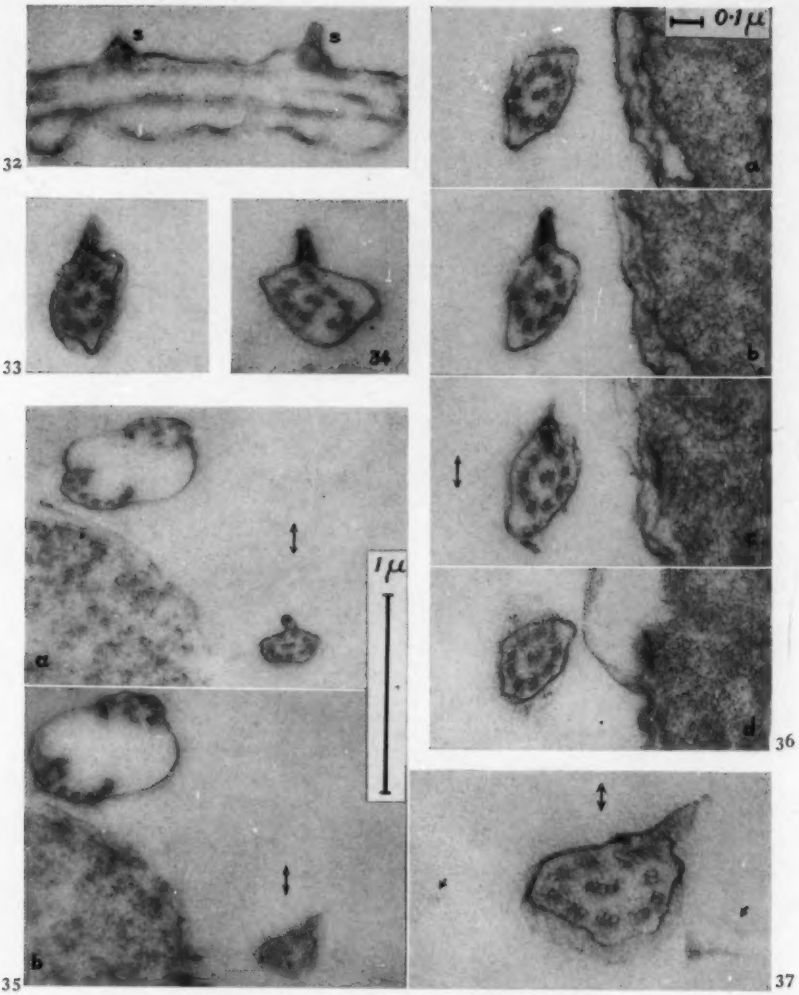


31 30



29

I. MANTON—PLATE VII



I. MANTON—PLATE VIII

sections with the position of the two central fibres; it is indicated by the double-headed arrow in both figures.

A more elaborate demonstration is provided by the series of four successive sections reproduced in Figs. 36*a–d*. The two central sections (Figs. 36*b* and *c*) contain the spine, which is symmetrically placed relative to the passage of the knife which seems likely to have been from below upwards as the sections lie on the page. In all four sections the symmetry of the organ is undoubtedly such that the median line, beginning at the spine, passes between but not through the two central strands. Two further specimens are included in Figs. 33 and 34.

Finally Fig. 37, a more highly magnified view of the flagellum of Fig. 35*b*, is added to show that the plane of bipartition in each of the nine strands of the peripheral ring is arranged radially as in other organisms. This picture also shows very faint traces (marked by arrows) of the two lateral lines of hairs, though the details of how these are arranged at their point of emergence from the flagellum requires more favourable material than this for effective study. The most that can be said is that the sections indicate that the hairs are indeed borne laterally, as was abundantly clear from the whole mounts.

Some evidence on the plane of symmetry of the flagellum relative to the rest of the cell has also been ascertained. Fig. 28*a–d* shows four sections selected from a longer series through a cell. The three lower sections (Fig. 28*b*, *c*, and *d*) show the functional flagellum and the blind base beside it; in Fig. 28*a*

PLATE VII

FIG. 26. A coiled cell showing nucleus (*N*), the two basal bodies, the root touching two mitochondria and sections of the coiled flagellum (arrows). R73B. ×20,000.

FIG. 27*a* and *b*. Two sections through a pair of bases and root; Fig. 27*a* shows both bases and a complex of fibres above the functional base, Fig. 27*b* nearer to the cell surface showing the vestigial base occluded and the band-shaped 'root' (*r*) near the functional base. E395.2 and E395.5. ×60,000.

FIG. 28*a–d*. Series of four sections through the two basal bodies to show the mutual symmetry of the functional flagellum and vestigial base; the arrow in Fig. 28*a* indicates the median plane of the functional flagellum. E393.22, E393.6, E393.4, and E393.2. ×27,000.

FIG. 29. A similar specimen more highly magnified. The arrow shows the median plane of the functional flagellum. H2541. ×44,000.

FIG. 30. A coiled cell showing the vestigial base and part of the functional flagellum near a spine (*s*). R47B.

FIG. 31*a* and *b*. Two successive sections through a similar specimen showing two spines (*s*). H2497 and H2487. ×36,000.

PLATE VIII

FIG. 32. LS of a flagellum passing through two spines (*s*). S633.30. ×36,000.

FIGS. 33 and 34. Two different specimens in TS passing through a spine; allowing for displacement in cutting both have the plane of symmetry vertical to the page. H2507 and H2576. ×50,000.

FIG. 35*a* and *b*. Two successive sections through a flagellum in the region of a spine to show the nature of the displacement which occurs in cutting; in both, the plane of symmetry is vertical to the page. H2545 and H 2562. ×30,000.

FIG. 36*a–d*. Four successive sections through a flagellum in the region of a spine showing the symmetry more clearly. H2585, H2577, H2578, H2580. ×50,000.

FIG. 37. The section of Fig. 25*b* more highly magnified showing the two central strands and the radial position of the split between half strands more clearly. H2566. ×*c*. 80,000.

the flagellum is seen slightly farther out. In this section the plane of symmetry of the flagellum, as indicated by the position of the two central strands, is marked by the double-headed arrow. A similar specimen more highly magnified is reproduced in Fig. 29. In both, the plane of symmetry of the functional flagellum would pass through the second basal body if both were traced at the same level. This is the normal type of symmetry where cilia in plants are arranged in pairs, whether these are isokont (*Pandorina*, Manton, 1955*a*) or heterokont (*Synura*, Manton, 1955).

The vestigial plastid. The most important remaining cell inclusion is the vestigial plastid or, perhaps more correctly, the body which is thought to be of this nature. This is labelled *p* in Fig. 5, Pl. II. It is a body about the size of a mitochondrion but with quite different contents. Instead of the tubular villi there is a mixture of large, rather uniform angular granules, and vesicles of various sizes. Only one such organ can normally be found in a cell and it is not associated with a flagellum. There is nothing else at all likely to represent the 'eyespot' of Lloyd Williams and though anyone familiar with *Fucus* (Manton and Clarke, 1956) will at once know that this is not an eyespot in the accepted sense, a reduced and vestigial chromatophore it could certainly be.

Other cell inclusions. As may best be seen in Fig. 2, Pl. I, the cell in addition contains apparently empty vesicles of various sizes, a few small fat bodies (two are contained in this figure) and doubtless a very small amount of granular cytoplasm between the vesicles. No other organelles have been identified.

DISCUSSION

Apart from the various unusual features associated with the flagellum but not necessarily with the sexual function, the spermatozoid of *Dictyota* is a relatively primitive type of male gamete in the sense that, except for the vestigial plastid, it could scarcely be distinguished from a zoospore (cf. *Scytosiphon*, Manton and Clarke, 1957). That it is also undoubtedly descended from a biflagellate ancestor seems proved by the persistent presence of a second basal body, not visible externally, which appears to be also a functionless vestigial organ.

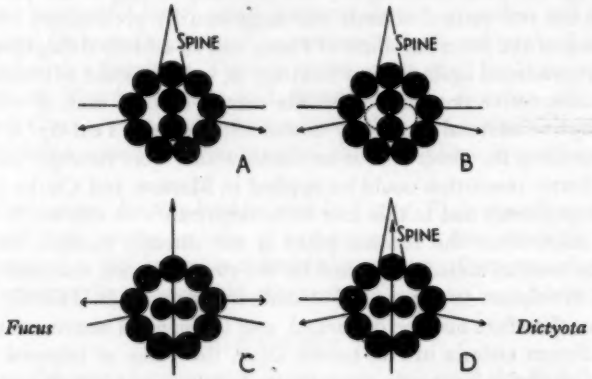
The conspicuous fibrous 'root' associated with the base of the actual flagellum but not with its functionless fellow is an organ of considerable botanical interest. Its position in relation to the flagellar base, together with its fibrous nature, suggests a possible homology with the so-called proboscis of *Fucus*, an organ which hitherto has been unexplained and without parallel. If this suggested homology is genuine it is necessary to believe that *Dictyota* is in the more primitive condition although in neither case is there any experimental evidence to guide one. The close association of the root in *Dictyota* with certain mitochondria suggests nevertheless the possibility of some respiratory function in connexion with the flagellum. Such a suggestion would be entirely new if applied to *Fucus* though it is one which could profitably be examined.

The flagellum itself has proved to be at least as informative as was hoped though in more ways than could have been foreseen. The question of flagellar symmetry with which the inquiry started has been answered with clarity and, one may hope, finality. That a cilium or flagellum, even without appendages and cylindrical in shape, is nevertheless essentially a bilaterally symmetrical organ has been known since the numerical facts of the 9+2 fibre pattern were first discovered (Manton and Clarke, 1951); discussions will be found in Manton, Clarke, and Greenwood (1953) and in Manton (1955*a*). When *Dictyota* itself was studied without the aid of sections (Manton, Clarke, and Greenwood, 1953) the evidence from spontaneously dismembered specimens was pieced together to give a tentative diagram reproduced again here as Text-fig. A. That this diagram was probably wrong in the position attributed to the two central strands was suggested by preliminary observations on sections of the front flagellum of *Fucus*, and an emended diagram (Manton, 1955*a*), reproduced again here as Text-fig. B, was proposed to record the most obvious alternative possibilities for the central strands each of which would give complete bilateral symmetry to the whole organ. Text-fig. C is the diagram recording the observations on *Fucus* which were strongly confirmed as soon as better resolution could be applied in Manton and Clarke (1956); the plane of symmetry had in this case to be determined in relation to the lateral lines of hairs since the median plane is not directly marked. In *Dictyota*, where the median strand is marked by the row of spines, the evidence for its position in relation to the central strands is expressed in Text-fig. D. Text-fig. A can therefore now be discarded, and the general statement made that, using different criteria in two brown algae, the plane of bilateral symmetry has been directly determined as passing between but not through the two central strands. This generalization is probably true for cilia and flagella of plants and animals as a whole.

While the precise position of the lateral hairs in terms of adjacent strands or adjacent spaces between strands has still to be ascertained on more favourable material, the flagellum of *Dictyota* has yielded two other unexpected facts each of considerable interest from a developmental point of view. It has long been known (cf. Greenwood, Manton, and Clarke, 1957 for *Vaucheria*, Manton and Clarke, 1957 for *Scytosiphon*) that the nine strands of the peripheral ring pass through the diaphragm separating the flagellum proper from its basal body and are continued into the wall of the basal body itself; from this observation alone, however, it cannot be known whether this description expresses a developmental fact concerning the differentiation of a basal body or whether the order of events occurs the other way round. The observation that even a functionless basal body contains nine fibres in its wall is conclusive evidence that the latter is indeed the case. The fundamental problem of why there should be 9+2 fibres in all cilia and flagella with such unfailing regularity throughout plant and animal kingdoms has sometimes been thought of as denoting some dynamic necessity connected with the functioning of the adult organ. This may well be true but it is clear that we must now also

look for a developmental explanation of why there should be nine fibres in the wall of a basal body before the flagellum itself is formed. This raises entirely different considerations from those involved in flagellar motion.

The origin of the flagellar membrane by uplift of the cell-body membrane as the flagellum uncoils is an unexpected observation which may be of considerable importance in revealing new properties in structures which, because of their appearance when dead, are given names derived from the macroscopic world but which at these much smaller sizes may prove to have significantly different physical properties. No macroscopic membrane would be expected to display a capacity for plastic deformation on the scale found in this case



Diagrams illustrating the history of knowledge of flagellar symmetry in brown algae; the pairs of lateral lines in each case indicate alternative positions for the lateral hairs, arrow heads being attached to the positions most probable for the organism concerned at the time of publication of the diagram. Fig. A, *Dictyota* reconstructed without the aid of sections, from Manton, Clarke, and Greenwood (1953). Fig. B, Two alternative possibilities for the central strands each giving complete bilateral symmetry to the whole organ, from Manton (1955*a*). Fig. C, Front flagellum of *Fucus* as found in sections, with the central strands related to the lateral hairs, from Manton (1955*a*). Fig. D, *Dictyota* in section, with the central strands related to the position of a spine; this diagram can now replace Fig. A.

unless it possessed powers of flow of the type normally associated with a liquid. In the macroscopic world, however, a liquid membrane would normally be regarded as a tautology since tensile strength of an order above that of a normal liquid is usually implied in the use of the word membrane. That these distinctions may no longer exist at the scale of sizes involved in some at least of the objects seen by electron microscopy is probable, and though full discussion here would overload the present communication it is nevertheless perhaps of importance to draw attention to this particular observation since it gives rather striking ocular demonstration of physical properties which may well be shared by other so-called membranes in different parts of the cell.

ACKNOWLEDGEMENTS

Very grateful thanks must be extended to the owners of the microscopes used and especially to the authorities of the Philips factory at Eindhoven for their hospitality in the summer of 1956 and to Dr. K. R. Porter of the Rockefeller Institute for hospitality at Christmas in the same year. I am also greatly indebted to the staff of the Marine Station at Menai Bridge for facilities and much help in the collecting of material during repeated visits. Finally I am personally indebted to my colleague A. D. Greenwood for co-operating in every possible way during the critical days on which these annual attempts at fixation have been carried out and without whom the relative success which has been achieved might never have been reached.

LITERATURE CITED

GREENWOOD, A. D., MANTON, I., and CLARKE, B. (1957). Observations with the electron microscope on the structure of zoospores in Vaucheria. *J. Exp. Bot.* **8**, 71–86.

LLOYD WILLIAMS, L. (1897). Antherozoids of Dictyota and Taonia. *Ann. Bot.* **11**, 545–53.

—— (1898). Reproduction in Dictyota dichotoma. Ibid. **12**, 559–60.

—— (1905). Studies in the Dictyotaceae. III. The periodicity of the sexual cells in Dictyota dichotoma. Ibid. **19**, 631–50.

MANTON, I. (1955) Observations with the electron microscope on Synura caroliniana. *Proc. Leeds Phil. Soc.* **6**, 306–316.

—— (1955*a*). Plant cilia and associated organelles. In symposium on *Cellular mechanisms in Differentiation and Growth*. Princeton University Press. Ed. D. Rudnick.

—— and CLARKE, B. (1951). Demonstration of compound cilia in a fern spermatozoid with the electron microscope. *J. Exp. Bot.* **2**, 125–8.

—— —— (1956). Observations with the electron microscope on the internal structure of the spermatozoid of Fucus. Ibid. **7**, 416–32.

—— —— (1957). Observations with the electron microscope on the internal structure of the zoospore of a brown alga. Ibid. **8**, 294–303.

—— —— and GREENWOOD, A. D. (1953). Further observations with the electron microscope on spermatozoids in the brown algae. Ibid. **4**, 319–29.

Some Growth Regulators of Tobacco in Relation to the Symptoms of the Physiological Disease 'Frenching'

N. P. KEFFORD

Division of Plant Industry, C.S.I.R.O., Canberra, Australia

Received 23 March 1959

SUMMARY

Extracts of the shoot tips of normal and 'frenched' tobacco plants were chemically separated into acidic, neutral, and basic ether-soluble fractions. On chromatograms of these, some plant growth regulators were assayed using the *Avena* coleoptile section extension test.

Two acidic auxins and an acidic and a neutral growth inhibitor were found. One auxin, with the same R_F value as indole-3-acetic acid, was four times more concentrated in normal as in 'frenched' plants. No differences could be established between the two types of plants in regard to other growth regulators detected.

It is argued that the symptoms of the physiological disease 'frenching' could be explained in terms of an auxin deficiency.

INTRODUCTION

ALTHOUGH much is known of the biochemistry and of the physiological effects of the naturally occurring plant growth regulator indole-3-acetic acid (IAA), little progress has been made in relating the endogenous concentration of growth regulators to the growth of green plants. In this investigation, the auxin contents of healthy tobacco plants are compared with those of plants having the physiological disease called 'frenching'. This disease is not caused by a pathogen nor by a known deficiency of a mineral element (Wolf, 1957). The symptoms of 'frenching' in tobacco are: (i) inter-veinal chlorosis; (ii) narrow leaves, variously described as strap, sword, or string; (iii) failure of internodes to elongate; and (iv) shooting of lateral buds (i.e. a loss of apical dominance) (Wolf, 1957).

The known properties of auxin make it possible that an auxin deficiency is responsible for the development of 'frenching' symptoms in tobacco, particularly in regard to symptoms (iii) and (iv) noted above.

MATERIALS AND METHODS

Tobacco (*Nicotiana tabacum* L. 'Virginia Gold') was planted in the field at Northcliffe, Western Australia, in October and by the end of January some

plants were showing 'frenching' symptoms of varying degrees of severity; other plants in the same field were normal. Samples were taken from three categories of plants: normal, 'frenched' (average), and severely 'frenched' plants, and in all cases plants showing flower buds were avoided. On the severely 'frenched' plants, stem extension had ceased and the leaves were narrow and had developed little more than a main vein; the average 'frenched' plants were shorter than normal, but some stem extension still occurred; the leaves were narrower than normal. The leaves from all 'frenched' plants showed the characteristic inter-veinal chlorosis.

From 'frenched' plants, the upper four visible internodes (lengths greater than 3 mm.), including apex, leaves, and stem, were collected. From normal plants two samples were obtained by cutting below the upper 4 visible internodes as before, and from this piece the oldest leaf and internode were removed (termed normal 4th internode). The remaining 3 internodes together with leaves and growing point were called normal tip.

Immediately on harvesting, bulked samples (up to 2 kg. from 200 plants) were weighed, packed into absolute alcohol, and dispatched from Perth to Canberra. On arrival, samples were cooled to 0°, then macerated in the alcohol of transport. The macerated tissue stood at −10° for 24 hours. The alcoholic extract obtained by centrifugation was treated according to the scheme in Table I.

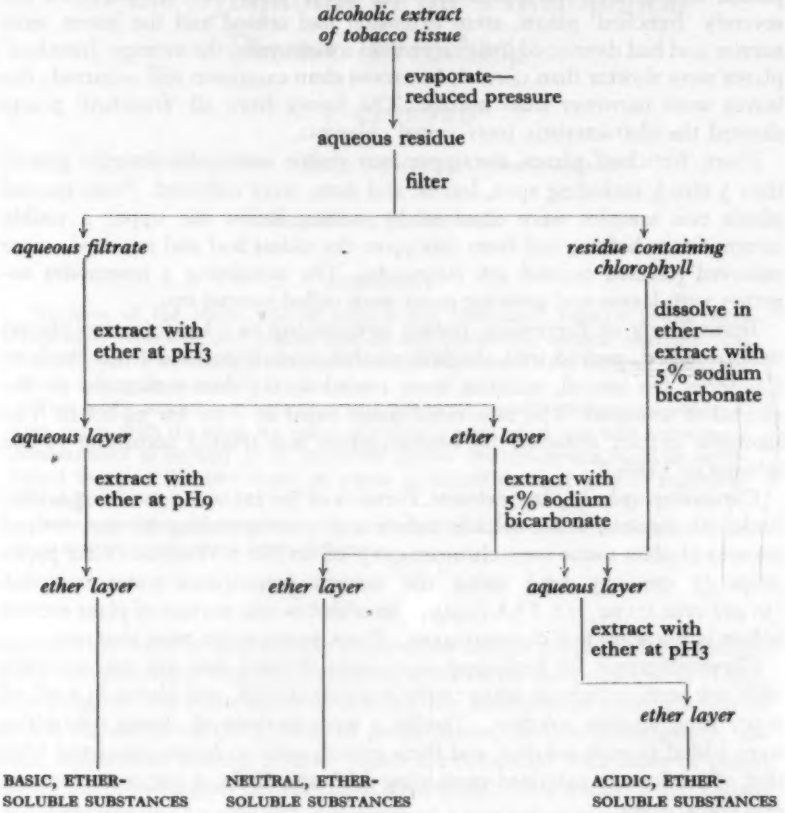
Chromatography of plant extracts. Portions of the extracts containing acidic, basic, or neutral, ether-soluble substances corresponding to the desired amount of plant tissue were chromatographed on No. 1 Whatman filter paper strips (3 cm.×35 cm.) using the solvent isopropanol:water:ammonia (30 per cent.)::10:1:1. IAA (3 μg.) was added to one portion of plant extract before application to a chromatogram. Blank paper strips were also run.

Chromatograms for biological assay were divided into 2·5 cm. sections, with one section corresponding to the position of IAA, and placed in 2 ml. of 2 per cent. sucrose solution. Twelve 3 mm. sections of *Avena* coleoptiles were added to each solution and their growth over 20 hours compared with that of sections in solutions containing IAA standards, 2 per cent. sucrose, and blank paper.

Checking methods. The general methods (Kefford, 1955 *a*) have now been used by a large number of investigators, but some variations of procedure required checking. Some samples of No. 1 Whatman filter paper contain growth inhibitors; in the present experiments a non-inhibiting sample was used. The plant tissues used in this investigation were held in alcohol at air temperatures for up to 32 hours before extraction, and it was shown, for similar tissues grown locally, that no losses in growth regulator activity occurred as a result of this treatment. As the R_F value of IAA is affected by other plant constituents, IAA guides were always run with a portion of plant extract. Chlorophyll residues contained more than 1/10 of the IAA in the plant extract (equivalent to 1·7 μg./kg. wet weight of normal tobacco tissue), hence the necessity for the treatment shown in Table I.

TABLE I

The scheme for the separation of the alcoholic extract from tobacco tissues into fractions containing acidic, basic, and neutral, ether-soluble substances



RESULTS

Acidic ether-soluble growth regulators in tobacco extracts. In Fig. 1 are given the results of *Avena* coleoptile section extension assays (expressed as percentage extension over original length) of chromatograms of the acidic ether-soluble substances from the tobacco samples: normal tip, 43 g.; normal 4th internode, 23 g.; 'frenched' tip, 60 g.; severely 'frenched' tip, 78 g. The same general pattern of growth activity was noted on each chromatogram: an area of weak growth promotion at R_F 0·0 to 0·2, the principal area of growth promotion at R_F 0·25 to 0·35 and an area of growth inhibition at R_F 0·4 to 0·65. Thus any differences in the acidic growth regulator status of these samples appeared to be quantitative rather than qualitative. Attention was directed to

the major area of growth activity at R_F 0·3, which is the area of the same R_F value as IAA. There is at present doubt about the identity of the tobacco auxin with this R_F value (Kefford and Helms, 1957; Vlitos and Meudt, 1957), but an exact identification of the growth regulators is not necessary for the

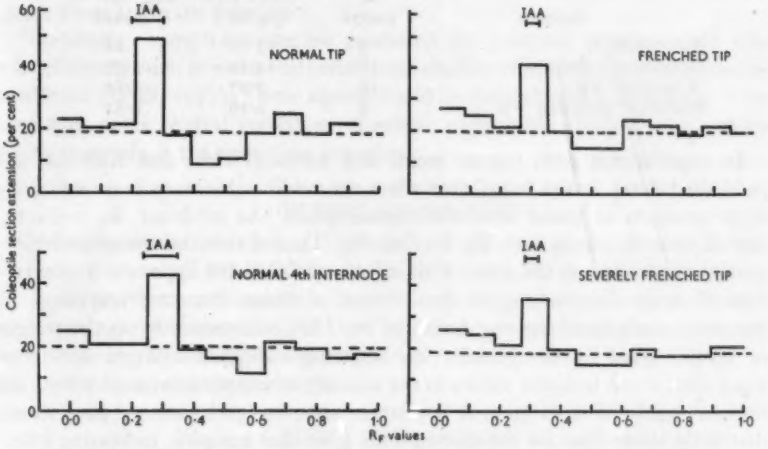


FIG. 1. Activities in coleoptile extension, shown as histograms, of squares cut from chromatograms of acidic, ether-soluble substances from extracts of the portions of normal and 'frenched' tobacco plants indicated. The horizontal broken lines represent the extension of controls and the positions of IAA marker spots are shown.

conclusions that are derived from these results. For convenience, growth activities detected at R_F 0·3 have been expressed as the concentration (μg./kg. wet weight) of IAA to which they are equivalent. Means of such concentrations are given in Table II for two normal and two 'frenched' samples.

On the basis of the variation in assays of the bulked material from each of the four plant sources, the difference between normal tip and normal 4th internode was statistically non-significant, while the differences between either of these and 'frenched' tip or severely 'frenched' tip was highly significant. The difference between the 'frenched' and severely 'frenched' samples was also significant. The assay error of the bulked material under-estimates the error appropriate to the comparison of individual plants, but it is probable, in view of the large difference between normal and 'frenched' plants and the continuing trend from average to severe, that the fall in IAA concentration with 'frenching' is significant on the basis of individual plants.

The substances responsible for auxin activity at R_F 0·1 and the inhibitory activity at R_F 0·5 have not been identified and are poorly characterized (Kefford, 1955 *a*; 1955 *b*). Estimates of relative amounts of these substances in extracts were therefore of doubtful value, but there was a suggestion of less inhibitor in 'frenched' plants.

TABLE II

Mean concentrations of auxin (expressed as μg. equivalents of indole-3-acetic acid (IAA) per kg. of wet tissue) in samples from normal and 'frenched' tobacco plants

Sample	Number of assays	Mean IAA (μg/kg.)	Estimate of std. dev. of assays
Normal tip	9	13·72	3·20
Normal 4th internode . .	18	14·19	5·53
'Frenched' tip . . .	16	3·27	0·82
Severely 'frenched' tip . .	6	2·16	0·81

In experiments with tomato stems and leaves (Helms and Kefford, unpublished data), it was found that when the acidic substances from relatively large amounts of tissue were chromatographed, the inhibitor, R_F 0·4–0·65, 'tailed' into the auxin area, R_F 0·3 (see Fig. 1), and reduced the growth promotion detectable in the area. This effect results in the apparent concentration of auxin diminishing as the amount of tissue extracted increases. A statistical analysis of the regression of the IAA concentration on the weight of sample used in the present investigation indicated a slight and non-significant trend to lower values as the size of the sample increased within the limits roughly of 10 to 50 g. A few larger samples (70 to 200 g.) gave values distinctly lower than for the corresponding smaller samples, indicating interference probably similar to that found with tomato extracts.

Neutral and basic ether-soluble growth regulators in tobacco extracts. On chromatograms of neutral, ether-soluble substances from all samples, a growth inhibitor was found at R_F 0·8 to 0·9. Several tests on extracts equivalent to 30 g. and 45 g. of the four tobacco samples showed no apparent differences in the amounts of this inhibitor, which was also shown to inhibit growth at all concentrations.

Growth inhibition, thought to be due to nicotine alkaloids, was detected on chromatograms of the basic, ether-soluble fraction.

DISCUSSION

The concentration of auxin was higher in both portions of the normal tobacco tissue than in the two types of 'frenched' tissue; no differences were detected in other plant growth regulators studied. Although no evidence presented here bears on any causal relationship between auxin concentration and the mode of growth of 'frenched' plants, the facts are compatible with the hypothesis that the stunted condition of 'frenched' plants is due to auxin deficiency. The leaf shape symptoms may also be due to an auxin deficiency, since Skoog and Miller (1957) show that auxin is probably a necessary factor in cell division. Comparisons of cell sizes in 'frenched' and healthy adult leaves (Wolf, 1935) and of transverse sections of adult 'frenched' and embryonic normal leaves (Wolf, 1935; Avery, 1933) suggest that cell division ceases prematurely in 'frenched' leaves.

The possibility that 'frenching' symptoms are due to a deficiency of a gibberellin is unlikely since Paleg (personal communication) observed that treatment of 'frenched' tobacco plants with gibberellic acid accentuated the symptoms. If gibberellins and auxins interact in the control of plant growth, the above observation could support the contention that 'frenching' symptoms result from an auxin deficiency.

'Frenching' symptoms can be produced by a variety of apparently unrelated agents such as some soil conditions, thallium toxicity, or sulphanilamide treatment (Wolf, 1957). These agents could be acting by each attacking, in its own way, some central growth mechanism, such as the auxin system, which reacts to produce the symptom complex.

ACKNOWLEDGEMENTS

The collection of material at Northcliffe, Western Australia, was organized by R. C. Rossiter, C.S.I.R.O., Regional Laboratory, Perth, and supervised by N. J. Halse, Western Australian Department of Agriculture, Perth; to both I am very grateful. I also thank L. A. T. Ballard for suggesting that auxin may be involved in 'frenching' symptoms, M. L. Dudzinski and G. A. McIntyre for statistical treatment to the data.

LITERATURE CITED

Avery, G. S. (1933). Structure and development of the tobacco leaf. *Amer. J. Bot.* **20**, 565–92.

Kefford, N. P. (1955 *a*). The growth substances separated from plant extracts by chromatography. I. *J. Exp. Bot.* **6**, 129–51.

—— (1955 *b*). The growth substances separated from plant extracts by chromatography. II. Ibid. **6**, 245–55.

—— and Helms, K. (1957). Indoleacetic acid in two species of *Nicotiana*. *Nature, Lond.* **179**, 679.

Skoog, F., and Miller, C. O. (1957). Chemical regulation of growth and organ formation in plant tissues cultured *in vitro*. *Symp. Soc. exp. Biol.* **11**, 118–31.

Vlitos, A. J., and Meudt, W. (1957). The auxins of Maryland Mammoth tobacco as separated by paper chromatography (A progress report). *Plant Physiol.* **32**, Suppl. xx.

Wolf, F. A. (1935.) *Tobacco diseases and decays*, 1st ed. (Duke University Press: Durham, North Carolina).

—— (1957.) *Tobacco diseases and decays*, 2nd ed. (Duke University Press: Durham, North Carolina).

The Metabolism of 3-Indolylacetic Acid in Plant Tissues

T. A. BENNET-CLARK AND A. W. WHEELER[1]

Department of Botany, King's College, London

Received 10 December 1958

SUMMARY

The nature of the metabolic products of 3-indolylacetic acid (IAA) extracted from potato tuber disks treated with aerated IAA solution has been investigated. Two major products, known at first as 'V' and 'P' in these studies, have been isolated and 'V' has been identified as 3-indolylacetylaspartic acid (IAcAsp).

The rapid uptake of IAA is inhibited by metabolic poisons such as 10^{-3} M. cyanide. The maximum mean internal concentration exceeds the external concentration in well-aerated cultures. The mean internal concentration, however, only remains high for a short period and then falls off rapidly as a result of extrusion of absorbed IAA into the external solution. This extrusion is not inhibited by 10^{-3} M. cyanide; when the mean internal IAA concentration is 150 μ mol/g. extrusion occurs into a solution of 700 μ mol/ml. and the localized IAA concentration must therefore exceed this value. We conclude therefore that the IAA concentration in the sites where it has accumulated exceeds the concentration of IAA outside.

Uptake of IAA and also its further conversion are inhibited by indolylacetonitrile and promoted by aspartate, but this promotion is not associated with any gain in amount of indolylacetylaspartate (IAcAsp).

The data suggest that IAcAsp may be formed in tissue from 'bound IAA' rather than free IAA.

The 'accelerator α' found in potato and beans which has similar R_F to IAcAsp has been shown definitely to be some other substance or substances and not IAcAsp as was at first thought possible.

INTRODUCTION

ANALYSIS of the effects of 3-indolylacetic acid (IAA) on plant extension growth should be accompanied by a study of its fate during the process. Ball (1953) showed that immersion of rhizomes of *Aegopodium* in IAA 10^{-4} M. for 45 minutes was followed by a characteristic growth response; there was at first an acceleration followed by a retardation. A somewhat similar behaviour was shown in *Avena* coleoptiles by Ball and Dyke (1956). The retardation was thought possibly to be due to conversion of IAA in course of its metabolism in the tissue into some inhibiting substance. The present study was begun to examine this question and products of metabolism of IAA (called here 'V' and 'P') had been isolated when Andreae and Good (1955) described 3-indolylacetylaspartic acid as a product of IAA metabolism. This was shown here to be the same substance as our 'V' which had not then been characterized.

[1] Present address: Department of Botany, Rothamsted Experimental Station, Harpenden, Herts.

This paper is concerned with certain aspects of the accumulation of IAA by plant tissues and of its subsequent fate.

ANALYTICAL METHODS

Growth substances were extracted from plant tissues or solutions by ether or ethyl acetate. Tissue samples were killed by boiling in distilled water and then cooled and macerated. The macerate was brought to pH 3 by H_3PO_4 and ethyl acetate was added. This was remacerated and after cooling to 0° C. and centrifuging the ethyl acetate was poured off. Two further extractions with ethyl acetate were made. The combined ethyl acetate extracts were then extracted with 5 per cent. $NaHCO_3$ three times and the $NaHCO_3$ solution, after bringing to pH 3 was extracted with ethyl acetate or peroxide-free ether.

Chromatography of the material extracted followed standard practice using *iso*propanol:ammonia (saturated solution):water; 9:1:1 or *n*-butanol:acetic acid (glacial):water; 4:1:1 as solvents. Quantitative estimations of amounts of material separated on chromatograms were effected by bio-assay using standard wheat coleoptile section tests (vars. Atle and Eclipse). Location of marker spots was effected by the ferric-perchloric, Salkowski, or Ehrlich reagents.

Colorimetric estimation of IAA in solution was done by mixing 1 ml. of the sample with 2 ml. of 0·01 M. $FeCl_3$ in 30 per cent. $HClO_4$ and measuring the extinction coefficient at 530 mμ after 90 minutes.

Colorimetry of the spots of indole derivatives on the paper chromatograms was effected as follows. The air-dried chromatogram was sprayed with $FeCl_3$–$HClO_4$ reagent and 24 hours later the coloured spots were cut out and put into 60 per cent. $HClO_4$ in a centrifuge tube. The paper disintegrates but the coloured complex is not destroyed and after centrifuging down the paper residues the supernatant is transferred to a spectrophotometer and the extinction coefficient determined as with the normal aqueous solution. The absorption at 530 mμ is close to the maximum for IAA, IAcAsp, and DIDAA[1] the calibration curves for which are given in Fig. 1. The extinction coefficient in 60 per cent. $HClO_4$ after elution from the paper remains fairly constant for about an hour but on longer exposures rises somewhat with IAcAsp and decreases with IAA.

Recognition of indole derivatives on paper chromatograms. Apart from colour reactions and R_F values, further checks on identity of esters, nitriles, and amides were made by hydrolysis of material located on paper chromatograms. The spots, located by u.v. fluorescence using a 'Hanovia' chromatolite, were cut out and placed in a pyrex tube with 2 ml. of N.NaOH or 7N.NaOH and heated in a boiling water bath for 1 hour. When cool, the mixture was brought to pH 3 with H_3PO_4 and extracted three times with ethyl acetate and, after concentration, placed on the starting-line of a chromatogram which was then run with *iso*propanol:ammonia.

[1] IAA: 3-indolylacetic acid; IAcAsp: 3-indolylacetylaspartic acid; DIDAA: the compound described (probably incorrectly, see p. 476) by Houff *et al.* as N:N^1-di-indolyldiacetic acid.

IAN and the methyl ester of IAA yielded IAA after hydrolysis with N.NaOH and amounts as low as 20 μg. of each could be recognized from paper spots by their original R_F values and colour reaction and their conversion by the hydrolysis procedure to IAA.

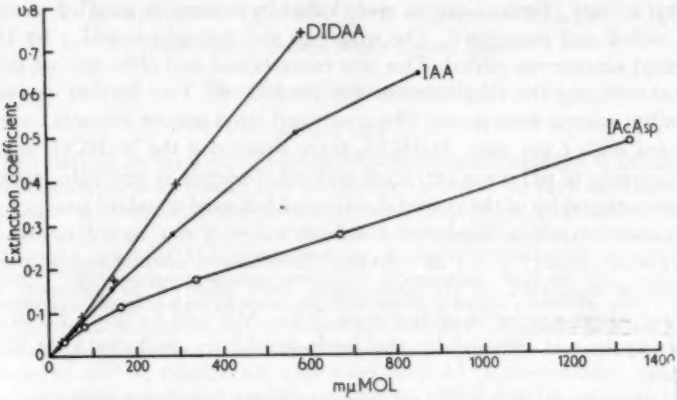


FIG. 1. Calibration data for indole derivatives eluted from spots on paper with $HClO_4$, the paper having been sprayed with $Fe/HClO_4$ 24 hours before elution. DIDAA has been assumed to have a molecular weight of 348.

Quantitative studies showed that recoveries of IAA after hydrolysis of spots on paper consisting of IAN, the methyl ester of IAA, or IAcAsp were of the order of 60 per cent. of the theoretical expectation both with N.NaOH hydrolysis and with the 7N.NaOH hydrolysis which is necessary when dealing with IAcAsp.

Acid hydrolysis was also carried out by eluting spots, concentrating under reduced pressure, adding 0·2 ml. of 2N.HCl, and heating for 1 hour on a boiling water bath. After removal of the HCl the material was transferred to paper for chromatography with *n*-butanol:acetic acid. Aspartic acid is liberated from IAcAsp and was recognized by ninhydrin spraying and identity of R_F value with a marker aspartic acid spot. IAA is completely destroyed by this process.

The development of these procedures for obtaining a further check on the identities of 'spots' which it was thought might contain nitriles, amides, or esters of IAA led to a further study of material that had been obtained from *Aegopodium* rhizomes and other plant material and was thought to be IAN on the basis of colour reaction and R_F (Kefford, 1953, 55). The isolation procedure in this present study was the same and colour reactions and bio-assays gave essentially the same results as described by Kefford. Alkaline hydrolysis of the spots, however, completely failed to reveal any liberation of IAA. As the yields are relatively good and the amounts of suspected or 'alleged IAN' were fairly large it must now be concluded that the previous identification of

the spot of R_F 0·8 (ca) as IAN is completely erroneous. This material which showed some biological activity may be compared with the material reported by Vlitos and Meudt (1957) though neither is as yet characterized.

A somewhat similar position was found regarding the 'accelerator α'. At first we thought it was highly probable that this might be indolylacetylaspartic acid. The R_F was similar and a further feature of similarity was the fact that the growth response after 5–6 hours was insignificant but was very marked with both 'α' and IAcAsp after 20–24 hours.

A chromatogram from bean shoots showing considerable 'α' activity was eluted and hydrolysed by 7N.NaOH, and the products of hydrolysis acidified to pH 3 extracted and run again as a second chromatogram. No activity in the IAA region was detected. Had IAcAsp been present a strong reaction would have been obtained. Clearly this shows that little IAcAsp is present normally and that 'α' is definitely some other substance.

Materials. Most of the work dealt with concerns accumulation of IAA and its fate in disks of potato tissue (var. King Edward). Tubers were scrubbed clean and surface sterilized with 0·05 per cent. $HgCl_2$ and disks 3·5 cm. diameter and 0·65 mm. thick were cut from the internal tissue. Batches of disks were washed and aerated overnight in sterile distilled water before 'feeding' with IAA.

After various times of 'feeding' the amount of IAA removed by the tissue from the external solution was estimated and the tissue itself was boiled to destroy enzymes as described, macerated, and analysed for growth substances or their derivatives or metabolic products by the methods described.

Certain treatments with metabolic or enzyme inhibitors are described.

EXPERIMENTAL RESULTS

Uptake of IAA by aerated potato disks

One hundred potato disks, fresh weight 50 g., prepared as described, were aerated in sterile distilled water overnight to wash away detritus. They were then aerated in 500 ml. of IAA, 100 mg./l. Samples were removed at intervals for analysis and the results are shown in Fig. 2. It will be noted that there is a very rapid initial uptake of IAA by the tissue. The mean concentration in the tissue rises from near zero to 675 mμ mol/g. tissue; this causes a drop in the external concentration from 530 to 385 mμ mol/ml. This high internal content of IAA falls off quickly and this is accompanied by a rise in the external concentration which, however, falls off slowly after about 6 hours.

In Fig. 3 are plotted the ratios of the 'mean internal concentration' of IAA to the external concentration. This is the function generally described as an accumulation ratio. As seen, this rises quickly to a peak value of about 1·75 with external IAA at 100 mg./l. and then falls rapidly to about 0·1.

This decrease in quantity of internal IAA from its peak value is clearly an important and complex phenomenon. The data of Fig. 2 show incidentally that the loss of IAA is accounted for only in small part by conversion to meta-

bolic products. The loss of 450 mμ mol/g. tissue between the 1st and 8th hour is accompanied by formation of only about 12 mμ mol/g. of the recognized indole derivative IAcAsp and an uncharacterized substance (termed 'P') giving instantaneously a pink colour with the ferric perchloric reagent, which is almost certainly an indole derivative. The greater part of the loss from the tissue between the 1st and the 6th hour is accounted for by extrusion of IAA

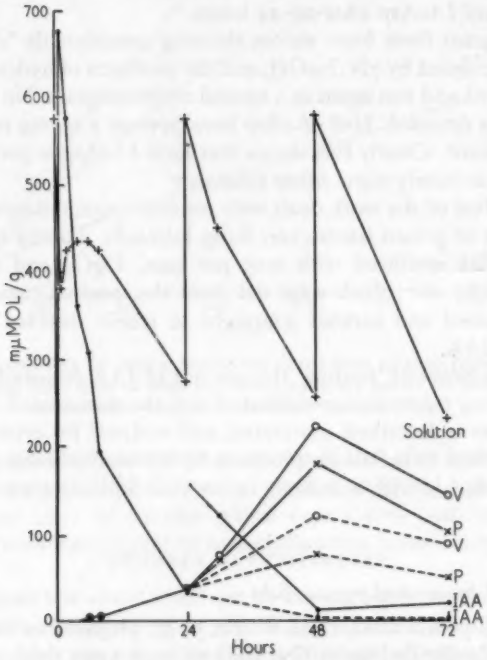


FIG. 2. Uptake of IAA by 50 g. potato disks aerated in 500 ml. of IAA (570 μM.). IAA in solution (—+—), in tissue (—•—), IAcAsp (—o—), 'P' (—×—). After 24 hours half the remaining disks were washed and aerated in water (shown by broken lines). The other half were supplied with further 500 ml. 570 μM. IAA, and after further 24 hours were washed and supplied with 500 ml. fresh 570 μM. IAA. Quantities of indole derivatives are given as mμ mol/ml. or mμ mol/g. fresh wt. of tissue.

from the tissue into the external solution, the IAA concentration of which rises quite considerably. From the 8th to the 24th hour there is further formation of IAcAsp and 'P' accompanied by further loss of IAA from the tissue and considerable loss from the solution which may in part be due to further metabolic oxidation of the IAA either by the potato tissue or more probably the start of activity of epiphytic bacteria.

The effect of metabolic inhibitors on the uptake and output phases of IAA

'accumulation' were separately studied in a number of experiments. Cyanide almost completely inhibits uptake of IAA by disks of tissue as shown in Fig. 4A. The graphs illustrate uptake of IAA from cyanide-containing solution and from control solution without cyanide which were shaken but not aerated by bubbling air through it as aeration would have carried away HCN

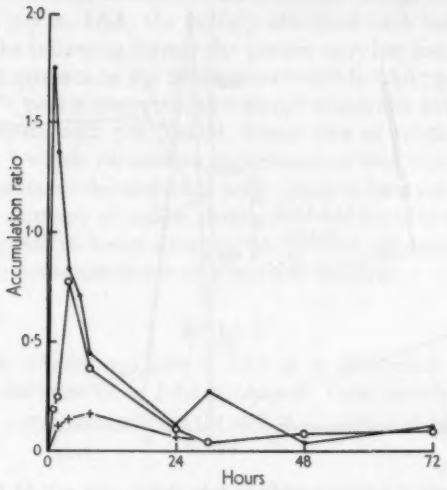


FIG. 3. The apparent or mean accumulation ratio of IAA from 570 μM. IAA (100 p.p.m.) —•—; 116 μM.IAA —o—; and 29 μM.IAA —+—. This ratio equals quantity per gr. fresh wt. tissue/quantity p. ml. solution.

from the cyanide-treated sample. The shaken cyanide-free control did not take up as much IAA as the well-aerated similar disks. It is clear that 10^{-3} M. cyanide virtually completely stops uptake of IAA by tissue and thus stops loss of IAA from the solution.

In other experiments active uptake of IAA by well-aerated (bubbled) disks was allowed to proceed for 2 hours (see Fig. 4B) so that disks were 'filled up' with IAA. The record of the experiment illustrated in Fig. 4B shows that slight reduction in concentration of the external IAA solution accompanied the intake by the tissue during this 2-hour period; the external solution was then changed. In the experiment of Fig. 4B 160 disks weighing 8 g. were aerated for 2 hours in 300 ml. solution (100 p.p.m.) containing 165 μmol IAA. At the second hour 60 disks (3 g.) were transferred to 20 ml. IAA solution (100 p.p.m.) and a second sample of 60 disks to 20 ml. IAA (100 p.p.m.) containing KCN (10^{-3} M.). These flasks were shaken and samples of disks and solution removed for analysis after 6 and 24 hours.

Loss of IAA from disks to solution takes place at approximately the same rate in presence and absence of HCN. The increase in quantity of IAA in the

20 ml. of external solution during the period from 2nd to 24th hours approximately equals the amount taken up by the tissue and then released again.

As the release into a solution of concentration 100 p.p.m. takes place as quickly in the presence of 10^{-3} M. HCN as when it is absent, one is justified

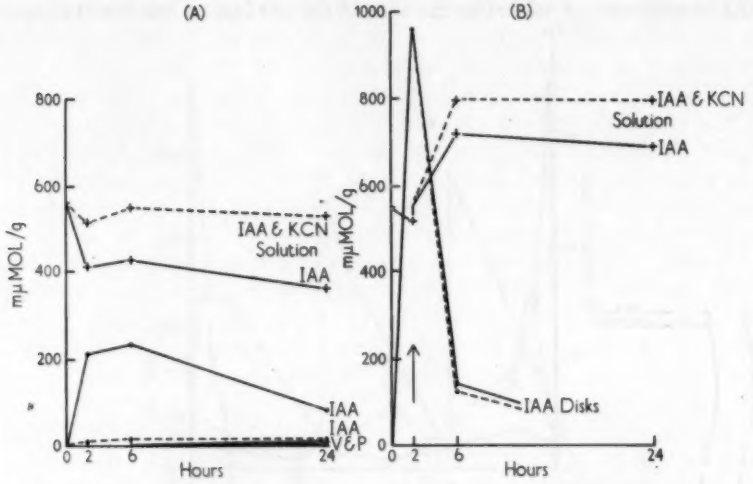


FIG. 4A. IAA contents of solution and tissue disks shaken with 10^{-3} M. KCN (broken lines) and controls without KCN (continuous lines). Note negligible production of IAcAsp and 'P' (—+—).

FIG. 4B. IAA contents of solution and tissue aerated with stream of air for 2 hours (8 g. disks in 300 ml. 570 μM. IAA). At the second hour 3 g. disks were transferred to 20 ml. 570 μM. IAA (——) and 3 g. to 20 ml. 570 μM. IAA + 10^{-3} M. cyanide (– – –). See text.

in concluding that this release is not a secretion process dependent on metabolic energy and that the concentration gradient causing or allowing it is positive, i.e. the concentration at the site in the tissue which is holding the IAA is greater than 700 μ mol/ml. although the mean tissue content is only around 150 μ mol/g.

Returning to the experimental data given in Fig. 2, after the rapid drop in mean internal IAA content between the 2nd and 24th hour, one part of the disks was transferred to water and well aerated and the second portion was again supplied with IAA solution (100 p.p.m.) and also well aerated. Analytical results for the former are shown by broken lines and for the latter by continuous lines. Those fed with more IAA showed a rather limited initial uptake of IAA and 24 hours later were re-fed with IAA and at this stage were quite turgid. The free extractable IAA of the tissue remained low in concentration and during the feeding period from 12th to 24th hour considerable quantities of indolylacetylaspartic acid (V) and the uncharacterized substance (P) were formed. 220 mμ mol IAcAsp/g. fresh weight were produced in disks fed with

IAA compared with 120 mμ mol/g. in the unfed disks. In these latter approximately 85 mμ mol/g. are produced together with 'P' coincidentally with the loss of nearly 30 mμ mol/g. of free IAA from the tissue. It is clear therefore that a large fraction of this IAcAsp and 'P' has been formed from IAA bound in the tissue in such a way as to be non-extractable.

Other experiments have in fact shown that at the end of the first 24 hours' feeding with 100 p.p.m. IAA, the initially absorbed IAA has become partitioned between the following forms: the greater part has been extruded into the medium; part remains in the tissue as extractable IAA; part is converted to IAcAsp and 'P'; part is converted to material which like IAcAsp yields free IAA after hydrolysis with 7N.NaOH. Some idea of relative quantities is shown by Table I which records an experiment of this type. These results also give an indication of the analytical errors likely to be involved. During the first 8 hours the recovery of indole derivatives was from 86 to 92 per cent., but in the subsequent 16 hours a further 30 per cent. approximately was lost, possibly owing to commencement of growth of bacteria.

TABLE I

Loss of IAA from solution and gain of IAA or its derivatives by potato disks. 50 g. disks aerated in 500 ml. of IAA 100 p.p.m. Total quantities in solution or disks are given in μ mols assuming, in the case of 'P', that it is a dimer of mol. wt. 348

Time (hrs.)	IAA in solutn.	IAA free in disks	IAcAsp in disks	'P' in disks	IAA released from residue by 7N.NaOH	Total indoles accounted for	% lost
0	265	0	0	0	0	265	0
1	193	33·8	0	0	0	227	14
2	213	28·9	0	0	0	242	8
4	218	18·1	0	0	0	236	10
6	218	15·4	0·8	0·5	0	235	11
8	213	9·7	0·3	0·7	1·3	226	14
24	138	1·3	7·2	3·5	3·6	154	42

In addition to free IAA and IAA bound as the peptide with aspartic acid and IAA presumably oxidized to 'P' there is IAA bound into complexes from which it is released by hydrolysis with 7N.NaOH in a boiling water-bath.

In general, after 24 hours' feeding in addition to IAcAsp (V) another material with small R_F and yielding a similar violet spot on spraying with ferric-perchloric reagent is found. This was termed V_o. V_o on hydrolysis with N.NaOH yielded material with the same R_F and colour reaction as V and possibly identical with IAcAsp therefore. On hydrolysis with 7N.NaOH it yielded IAA and aspartic acid. The larger residual amount of 'bound IAA' set free by hydrolysis with 7N.NaOH may, it seems likely, be found in peptide linkages. The fact that this bound IAA appears to be the source from which IAcAsp is being produced (together with 'P') during the period from the 24th to 48th hour suggests the possibility that these peptides have an

aspartic acid N-terminal residue combined with IAA. This is under investigation at present in this laboratory.

The product 'P' which is regularly formed in potato disks has an R_F between that of IAA and IAcAsp. It gives instantly a red or pink colour with the ferric perchloric reagent which suggested comparison with the material described by Houff *et al.* (1954) as N:N^1-diindolyldiacetic acid (DIDAA) as the product formed by iodine oxidation of IAA in alcoholic solution (+NaOEt).

As prepared by us, a mixture consisting largely of unchanged IAA was produced by the iodine-oxidation process as described by Houff *et al.* Other material giving the colour reaction of the alleged DIDAA was readily separated on paper or cellulose columns. This material was used as a calibration standard (Fig. 1) and was thought possibly to be identical with the material produced in the tissue termed 'P' on grounds of identity of R_F and colour reactions. In an endeavour to clarify this we hoped that an identification could be achieved, and Professor Wain and Mr. G. Clarke of Wye College very kindly examined our material. This proved to be a mixture of unstable compounds troublesome to separate. Infra-red absorption data, however, showed very strong absorption of 3340 cm.$^{-1}$ characteristic of unbound secondary-NH-grouping. The DIDAA formula suggested by Houff *et al.* is thus most improbable. Details will be published by Professor Wain and Mr. G. Clarke. Further investigation of 'P', almost certainly also a mixture and devoid of biological activity, is not being continued.

Influence of certain substances on accumulation and metabolism of IAA.

Results shown concisely in Table II are representative of several experimental treatments. Indolylacetonitrile, which is not hydrolysed by potato-tissue slices, markedly inhibits the uptake of IAA and virtually completely inhibits its conversion to IAcAsp and 'P' so that one presumes that it is acting as an inhibitory metabolite-analogue. Kinetics of this inhibition were not studied. 2:4-D in contrast appeared to promote the process of extrusion of IAA so that after 24 hours none was left in the tissue and relatively smaller amounts of the aspartic peptide and 'P' had been formed.

Aspartic and glutamic acids and to a lesser extent α-naphthylacetic acid caused a very remarkable increase in the amount of IAA taken up during the initial 2-hour accumulation phase (cf. also Reinhold, 1958); this very large initial accumulation was correspondingly quickly lost again so that after 24 hours the free IAA content of disks was the same in amino-acid treated and control samples. The production of IAcAsp and 'P' was not influenced.

DISCUSSION

Accumulation mechanisms. The ratio of mean internal IAA content to external concentration is shown in Fig. 3. The two phases, an intake and an output phase, have been discussed in relation to influence of cyanide on these processes.

TABLE II

Accumulation of IAA in μ mols/g. fresh weight and formation of IAcAsp and 'P' by certain tissues and by potato tissue in presence of certain growth substances and amino-acids

Tissue and treatment	Free IAA after 2 hrs.	Free IAA after 6 hrs.	Free IAA after 24 hrs.	IAcAsp 24 hrs.	'P' 24 hrs.
Potato disks					
IAA 20 p.p.m. alone . .	17·7	14·2	1·9	8·7	1·6
IAA 20 p.p.m. + NAA 18·6 p.p.m.	30·8	15·8	2·2	8·8	1·8
,, + 2:4-D 22·1 p.p.m.	15·6	10·0	0	5·2	1·8
,, + IAN 15·6 p.p.m.	11·5	5·6	2·2	0	0
,, alone	17·6	14·6	2·0	13·2	2·3
,, + aspartic acid 13·3 p.p.m.	58·0	13·6	1·7	13·5	3·2
,, + glutamic acid 14·7 p.p.m.	49·0	20·0	1·6	13·1	3·1
Aegopodium rhizome tips					
IAA 20 p.p.m.	7·3	1·4	1·6	5·2	3·1
Alaska pea epicotyls IAA 20 p.p.m.	20·7	51·3	3·9	22·7	0
Wheat coleoptiles IAA 20 p.p.m.	11·0	5·5	4·2	3·9	0

The results should be compared with those of Blackman (1956) who showed that isotopically labelled 2:4-D was taken up by *Lemna* during an initial period and then released. These results have been extended (Blackman, Senn, Birch, and Powell, 1959).

Data of Fig. 3 show that when the external IAA concentrations are lower than 100 mg./l. (20 and 5 mg./l.) qualitatively similar effects are produced with markedly lower maximum 'accumulation ratios', which is probably the reverse of what one would have expected. Normally the lower the external concentration the higher is the accumulation ratio.

As has been pointed out, since the output of IAA is not inhibited by metabolic poisons and since it occurs into solutions of greater concentration than the *mean internal* concentration it follows that there must be localization of internal IAA in cell regions of limited volume such as cytoplasm only or cytoplasm+wall. A consequence of this probability is that estimates of the IAA contents of tissues (as indeed of contents of other constituents) may bear relatively little relation to the concentration in the site which is occupied or to the physiological effects with which they are being correlated.

The mechanism of this initial accumulation can hardly be guessed at present. The most difficult features to explain are the dependence on metabolism, the 300 per cent. increase in extent brought about by aspartic and glutamic acids, and the rapid turn round to export of the accumulated IAA. This last might be assumed to be due to some form of irreversible injury although signs of such injury are, at least in the early stages, completely lacking.

Essentially similar behaviour appears to have been found by Blackman, Senn, Birch, and Powell (1959) with *Lemna* in regard to the uptake of isotopically labelled phenoxyacetic acids. In this case when external concentrations were high the rapid initial uptake was succeeded by an output of the labelled acid, and output also occurs when mean internal concentration is exceeded by the external concentration (Blackman *et al.*, Fig. 2).

Behaviour of other tissues. Similar behaviour has been observed (see Table II) with *Aegopodium* rhizome tips, pea epicotyl sections, and wheat coleoptiles. The last two did not produce 'P' but IAcAsp was produced in all three and in all three the phenomenon of relatively rapid loss of internal IAA occurred. Most of this loss was due to extrusion into the external medium.

Formation of indolylacetylaspartic acid and 'P'. The formation of these substances has been shown, as one would expect, to be blocked by treatment with cyanide. The procedure adopted was to allow disks to accumulate IAA for 2 hours and then treat with cyanide, shaking to provide some aeration. IAcAsp and 'P' are not formed and the absorbed IAA is rapidly extruded.

Indolylacetonitrile also is found to inhibit production of IAcAsp and 'P'.

The formation of the IAcAsp, one would guess, is probably brought about by production of an indolylacetyl derivative having an acyl or mercaptide bond such as indolylacetyl-CoA, which might then react with aspartic acid. The feature of interest in this connexion is the production of IAcAsp and 'P' from disks which have been fed for 24 hours with IAA and have then been washed clean. When such disks (see Fig. 1) which contain only small amounts of free IAA are aerated in water, IAcAsp is formed. In that experiment, 90 mμ mol/g. of IAcAsp was formed and 40 mμ mol of indolyl radicles in the form of 'P' (assuming that 'P' is a dimer) from the available 32 mμ mol plus presumed bound IAA. This does not demonstrate, though it suggests, that the IAA may be bound in the form of peptide linkages. This is now under investigation here, using isotopically labelled material. Siegel and Galston's results (1953) are also suggestive in this connexion.

Conclusion. The major conclusion to be drawn is that initial absorption occurs by a process controlled or activated by metabolic energy and resulting in accumulation to relatively high concentration in a limited region probably to ten times the mean concentration in the whole tissue. This figure of tenfold is based on the continued output of IAA from tissue to solution when the *mean* accumulation ratio is 0·1. The volume of the cytoplasm might well be about 10 per cent. of the total volume (i.e. 10 ml. per 100 g. tissue) and it would therefore be a reasonable guess that the IAA accumulates in this region rather than the vacuole. The pH of cytoplasm is probably around 7 and the 'free' IAA thus might be present as ions or possibly as unstable complexes readily extractable.

ACKNOWLEDGEMENT

Our thanks are due to the Agricultural Research Council for a grant which made this work possible.

LITERATURE CITED

ANDREAE, W. A., and GOOD, N. E. (1955). The formation of indoleacetylaspartic acid in pea seedlings. *Plant Physiol.* **30**, 380.

BALL, N. G. (1953). The effects of certain growth-regulating substances on the rhizomes of Aegopodium podagraria. *J. Exp. Bot.* **4**, 349.

—— and DYKE, I. J. (1956). The effects of indole-3-acetic acid and 2:4-dichlorphenoxyacetic acid on the growth rate and endogenous rhythm of intact *Avena coleoptiles*. Ibid. **7**, 25.

BLACKMAN, G. E. (1956). Interrelationships between the uptake of 2:4-dichlorophenoxyacetic acid, growth and ion adsorption. In *The chemistry and mode of action of plant growth substances*. Ed. R. L. Wain and F. Wightman. Butterworths Scientific Publications, London.

—— SENN, G., BIRCH, W. R., and POWELL, R. G. (1959). Factors controlling the uptake of phenoxyacetic acids by Lemna minor. *J. Exp. Bot.* **10**, 33.

HOUFF, W. H., HINSVARK, O. N., WELLER, L. W., WITTWER, S. H., and SELL, H. M. (1954). The nature of an oxidation product of 3-indoleacetic acid. *J. Amer. Chem. Soc.* **76**, 5654.

KEFFORD, N. P. (1955). The growth substances separated from plant extracts by chromatography. I. *J. Exp. Bot.* **6**, 129.

REINHOLD, L., and POWELL, R. G. (1958). The stimulatory effect of indole-3-acetic acid on the uptake of aminoacids by tissue of Helianthus annuus. Ibid. **9**, 82.

SIEGEL, S. M., and GALSTON, A. W. (1953). Experimental coupling of indoleacetic acid to pea root protein in vivo and in vitro. *Proc. Nat. Acad. Sci. Wash.* **39**, 1111.

VLITOS, A. J., and MEUDT, W. (1957). The auxins of Maryland Mammoth tobacco separated by paper chromatography. *Plant Physiol.* **32**, xx.

The Uptake of Growth Substances

II. THE ABSORPTION AND ACCUMULATION OF 2:3:5-TRIIODOBENZOIC ACID BY THE ROOT AND FROND OF *LEMNA MINOR*

G. E. BLACKMAN AND J. A. SARGENT

Department of Agriculture, University of Oxford

Received 7 March, 1959

SUMMARY

The absorption by *Lemna minor* of 2:3:5-triiodobenzoic acid (TIBA), labelled in the 2 position with iodine-131, has been investigated. Under constant conditions of light (300 foot candles) and temperature (25° C.) the course of uptake by the whole plant is dependent on the concentration of TIBA in the culture solution (pH 5·1). Up to *circa* 1 mg./l. a high rate of uptake in the first 30 minutes is followed by a slower but steady accumulation during the next 4·5 hours. When the concentration is increased to 5 mg./l. uptake, at first rapid, falls off progressively so that after 1–2 hours the content of the tissues starts to decrease because TIBA moves out in to the external solution faster than it enters. The magnitude of this reversal from a positive to a negative rate of uptake, particularly for the roots, is even more pronounced at 10 mg/l.

When plants, first placed in labelled solutions for 2 to 2·5 hours are transferred to culture solution the loss of the labelled compound to the external solution, especially from the roots, is very rapid: the decrease in root content may reach 90 per cent. in the first 80 minutes. The initial rate of egress is little affected either by the hydrogen ion concentration or the presence of unlabelled TIBA in the external solution. Between 5, 15, and 25° C. the temperature quotients range from 1·29 to 1·70 for the root and 1·66–1·37 for the frond.

Comparable experiments on the initial phase of uptake during the first 20 to 30 minutes demonstrate that (*a*) the rates are greatly dependent on the external pH i.e. uptake is largely in the molecular form and (*b*) between 5 and 15° C. the rates are not temperature dependent but between 15 and 25° C. they are: the quotients for roots and frond are 2·6 and 2·0.

Comparisons of the course of uptake of normal plants and plants previously frozen in liquid air showed that at both 1° and 25° C. 'pre-frozen' fronds continue to accumulate TIBA and may contain at the end of 5 hours 8 times as much as control fronds. In contrast, the uptake by the pre-frozen roots is depressed. The addition of high (30–50 mg./l.) but not low (10 mg./l.) concentrations of indole-3-acetic acid (IAA) to the external solution also causes a striking accumulation of TIBA in the frond but uptake by the roots is not changed.

Lastly, the presence of TIBA in the external solution while not affecting the uptake of cerium-144 in the first 2 hours subsequently depresses uptake by the root but not by the frond.

These findings are compared with the similar pattern of uptake for 2,4-dichlorophenoxyacetic acid described in the previous paper. The nature of the processes controlling the inward and outward flow of TIBA, their location within the cell and their bearing on the physiological actions of auxins are discussed.

INTRODUCTION

In 1942 Zimmermann and Hitchcock first showed that applications of 2:3:5-triiodobenzoic acid[1] to the tomato brought about changes in leaf shape, loss of apical dominance, and the early production of flower clusters. Since then numerous papers have appeared on the properties of TIBA and some restriction must inevitably be made in summarizing the knowledge that has accumulated. The published literature falls roughly into three categories; the influence of TIBA on flowering, the effects on extension growth, and the induced changes in morphological and anatomical structure.

Galston (1947) working with the soyabean concluded that while under the conditions which favoured flowering (short days) more flowers were formed by plants treated with TIBA, under long days flower formation could not be similarly initiated. De Waard and Roodenburg (1948) confirmed that by treating tomato plants in the early vegetative phase the time of flowering could be advanced but Zimmermann and Hitchcock (1949) in an extension of their earlier investigations emphasized that while TIBA can cause the formation of flower initials, it does not control flowering. With *Xanthium*, Bonner (1949) was able to bring about flowering in long days with TIBA and to counteract this effect by applications of naphthylacetic acid (NAA); conversely in short days the suppression of flowering by NAA could be reversed by TIBA. With *Kalanchöe*, irrespective of day length, TIBA always inhibited flowering (Harder and Oppermann, 1952). Finally Gorter (1955) claimed that both in tomato and *Phaseolus* TIBA brought about increases in flowering and fruiting.

Galston (1947) also examined the influence of TIBA on extension growth and in the *Avena* curvature test no growth promotion could be detected. Indeed, when TIBA was applied along with indole-3-acetic acid (IAA) the degree of curvature induced by IAA alone was reduced if the molar ratio of TIBA to IAA exceeded 3:1. Two additional tests were employed by Thimann and Bonner (1948). In the pea test at low concentrations TIBA had some growth-promoting activity but this effect was lost if the tissue was first washed for 2 to 4 hours. When mixed with IAA, TIBA augmented both extension growth in coleoptile sections and curvature in intact coleoptiles of *Avena*, provided that the concentrations were low; at high concentrations the effect was inhibitory. On the other hand, De Waard and Floroschutz (1948) employing the *Avena* curvature technique, Snyder (1949) studying the rooting of *Coleus* cuttings and Weintraub, Brown, Nickerson and Taylor (1952) examining the abscission of *Phaseolus* leaves all concluded that TIBA was antagonistic to the action of added IAA. Nevertheless, the fact that TIBA together with other substituted benzoic acids will increase the extension growth of *Avena* coleoptiles (Muir and Hansch, 1951) shows that this compound exhibits the properties of both an auxin and an anti-auxin.

Åberg (1950, 1953, 1957) has undertaken extensive studies of the auxin

[1] In the rest of this paper the conventional abbreviation of TIBA will be employed.

relationships in roots and has reached the conclusion that, at low concentrations TIBA acts synergistically with the supra-optimal concentration of internal IAA to bring about increased inhibition of root growth. At somewhat higher concentrations, TIBA assumes an antagonistic role to the endogenous auxins and at still higher concentrations it is toxic. Street (1955) has also found that the extension growth of tomato roots is decreased by the presence of TIBA. On the other hand Audus and Thresh (1956) question the general concept that the level of IAA in roots is always supra-optimal. On the basis of a biological assay of endogenous IAA coupled with chromatographic separation, they concluded that many effects of TIBA can be accounted for in terms of a resulting reduction in the content of IAA and a direct toxic action at higher concentrations.

It has long been established that applications of IAA will bring about cell division and modifications in cell differentiation (e.g. Kraus, Brown, and Hamner 1936) and it was postulated by Wenck (1952) that TIBA, because of its attributes as an anti-auxin, should also induce histological effects. By applying TIBA to the apical bud of *Bryophyllum* it was found that the number of veins in the leaves were diminished and that this reduction could be partly offset by the simultaneous application of IAA. Subsequently Wangermann and Lacy (1953) demonstrated that with *Lemna minor* the results were different: venation was increased by TIBA. With *Zinnia elegans* Applegate and Hamner (1957) also found that foliar applications of TIBA and the corresponding chloro- and bromo-substituted compounds induced further vascular differentiation. In a further analysis of *L. minor* made by Sargent and Wangermann (1959) it was established that TIBA and IAA produced dissimilar patterns of venation and that the response to TIBA could not be nullified by IAA. Since the concentration of TIBA in the external solution and the time of treatment were both critical factors the next logical step was to follow the course of uptake and this was the genesis of the present investigation. Such studies seemed particularly desirable in view of the unusual features of the uptake of substituted phenoxyacetic acids by *L. minor* described in the first paper of this series (Blackman, Senn, Birch, and Powell, 1959).

EXPERIMENTAL METHODS

The general experimental techniques employed were similar to those in the first paper (Blackman *et al.*, 1959). Briefly, the same clonal population of *L. minor* used by Sargent and Wangermann (1959) was cultured in a Steinburg (1941) nutrient solution, modified to increase the buffering capacity by doubling or quadrupling the content of potassium dihydrogen phosphate. The stock population was grown in plastic (polystyrene) containers which were placed in a water bath thermostatically controlled at 25° C. The plants received continuous illumination from daylight fluorescent tubes suspended above the bath and so adjusted in height that the intensity reaching the fronds was 300 foot-candles.

In order to minimize both pH drifts and contamination from algae and

micro-organisms, every 2 days the plants, after thorough washing, were transferred to fresh solution. This culture solution and the experimental solutions, unless otherwise stated, were adjusted before use to pH 5·1.

Before an experiment was started, single fully expanded fronds were teased apart from plants of the appropriate stage of development and the single fronds returned separately to the water bath for at least 12 to 15 hours. This procedure was adopted because other experiments had shown that without an adequate period of recovery after the separation the plants exhibited a variable and higher rate of uptake due presumably to a greater penetration through the 'wound' until the tissue had healed. To achieve uniformity of selection only those fronds were taken where the first daughter frond emerging from the envelope just did not project beyond the periphery of the mother frond. This basis was chosen because this stage of development was the starting point for the investigations concerned with the effects of TIBA on venation.

TIBA labelled with ^{131}I in the 2-position was obtained from the Radiochemical Centre, Amersham, and this, as the sodium salt was added to the culture solution to give concentrations ranging from 0·525 to 10 mg./l. In order to avoid all but minor changes in the external concentration due either to uptake or to evaporation there were only five plants per 200 ml. of solution contained in a glass beaker. The beakers were placed in a water bath and for most of the experiments the plants were subjected to the same light and temperature conditions as the stock population.

After the selected experimental interval, the plants were withdrawn from each beaker, washed for a few seconds in two lots of distilled water, blotted with filter-paper and the roots quickly dissected from the fronds. The five fronds and their roots were then placed in separate nickel planchettes in such a way that there was no over-lapping. The planchettes were put under an infra-red lamp and when dry counted under constant geometry with an end window counter. On general grounds it was considered that the errors due to self-absorption would be negligible. The tissues were thin, uniformly spaced on the planchette, and no difference in count was obtained for fresh or dried samples.

In experiments concerned with the effects of unlabelled TIBA on the uptake of cerium-144 the same methods were followed.

The fate of the (labelled) TIBA after entry was examined by chromatographic techniques. Batches of 300 fronds were first ground with a minimum amount of 0·1 N HCl in a glass grinding tube and the contents centrifuged. The supernatant liquid after concentration on a water bath constituted the 'acid' extract. Since the cell debris exhibited considerable radioactivity it was re-extracted with ether, centrifuged, and the supernatant liquid (after concentration at room temperature) will be termed the ether re-extract. After the second extraction the cell debris exhibited little activity.

All chromatograms were run on the same sheet of acetic acid washed Whatman 3MM filter-paper and the origin of each was confined to a 2 cm.

starting-line. The solvent employed was iso-propanol, water, and ammonia (S. G. 0·88) in the ratio by volume of 10:1:1. The paper was equilibrated with the vapour before running and the front was allowed to travel some 30 cm. After drying the paper, each chromatogram strip was cut out and placed in an electronic scanning device designed in the Department. This instrument records continuously the level of radio-activity along the length of the paper —see Loughman and Martin (1955).

EXPERIMENTAL RESULTS

The course of uptake. The inter-relationships between uptake, the external concentration, and the time of exposure were examined in a number of experiments. The results obtained at low concentrations are given in Fig. 1. Considering first the whole plant it is evident that both concentration and time

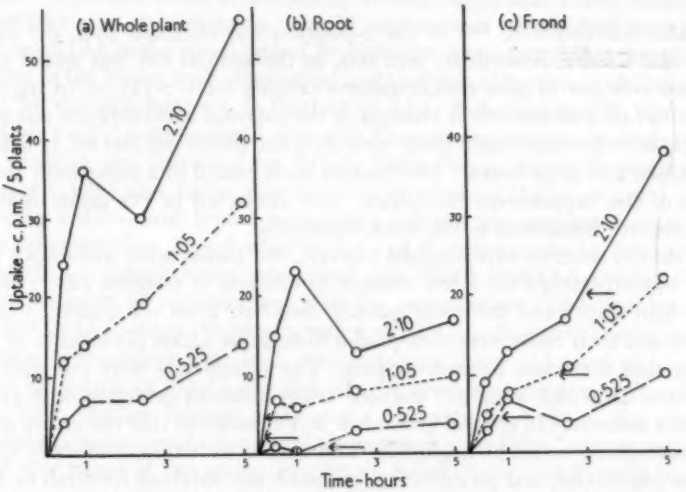


FIG. 1. *Expt. 1.* The course of uptake of TIBA from concentrations of 2·1, 1·05, and 0·525 mg./l. In this and subsequent figures the horizontal arrows indicate when the calculated mean internal concentration equals the external.

bring about a progressive accumulation. The trends for the frond are similar but the pattern for the root is different. In the first half hour accumulation at all concentrations is rapid, then there is a second phase when either[1] there is no further accumulation or for 2·1 mg./l. there may be a significant fall in the content which, in turn, is followed by a third phase when a significant accumulation at a slow rate again takes place. Since the loss from the roots appears to be greatest at the highest concentration, it is not surprising that when the range of external concentration is extended to 10 mg./l. the inter-

[1] Because the data include values of 0 (0·525 mg./l. after 1 hour), the transformation of $\sqrt{(x+\frac{1}{2})}$ was applied to the counts before statistical analysis was undertaken.

acting effects of time and concentration became more marked—see Fig. 2. From concentrations of 5 and 10 mg./l. uptake by the whole plant is at first rapid but eventually this process is reversed and there is a net loss from the plant. It would seem that this loss is determined by the balance between the opposing changes in the amounts contained in the root and frond. While the amounts in the frond continue to rise slowly during the course of the experiment, the amounts in the root, after reaching a maximum value, fall sharply.

When the experimental period is extended to 54 hours and allowance is

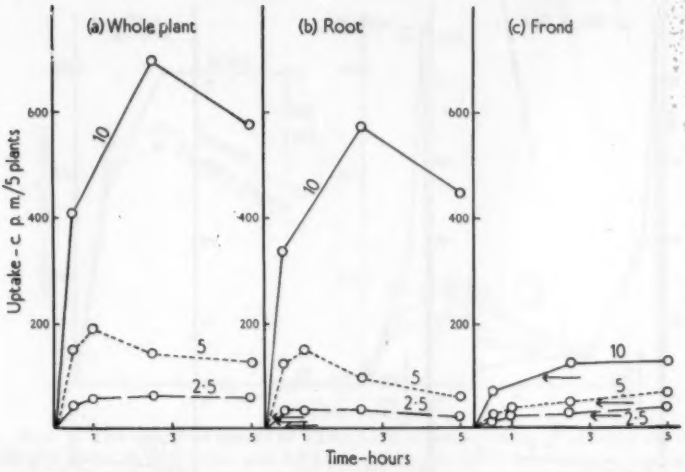


FIG. 2. *Expt. 2.* The course of uptake of TIBA from concentrations of 10, 5, and 2·5 mg./l.

made for the progressive increase in weight with time it is apparent from Fig. 3 that the changes in content are even more striking. For an external concentration of 10 mg./l. there is a high rate of accumulation by the root during the first 2 hours which is then succeeded by a progressive fall in content up to the end of the experiment. In contrast, the level in the frond, which is much less than that in the roots, steadily increases with time. For both root and the the whole plant the interactions between time and concentration are highly significant. At the concentration of 2·5 mg./l. the effects of duration of treatment are far less evident. As in Expt. 1, there is some suggestion that accumulation in the root can be resumed after a period of loss. In this instance the fall between 2 and 6 hours is replaced by a net gain in the next 48 hours.

At both concentrations during the experiment the plants made equal and considerable growth. After 2, 18, and 54 hours the mean dry weight per plant was 0·135, 0·165, and 0·266 mg. while the corresponding figures for the frond and root were 0·121, 0·139, and 2·25, and 0·014, 0·026, and 0·041 mg. respectively. Thus on an absolute scale, accumulation of TIBA with time in the frond is accentuated and this also holds for the root between 6 and 54

hours at the lower concentration. In contrast at 10 mg./l. though there is a net loss from the root between 2 and 18 hours there is a gain between 18 and 54 hours.

Since the fresh and dry weights of the frond and root were determined and if it is assumed that the total cell volume, excluding inter-cellular spaces, is approximately equal to the difference in fresh and dry weight, then the time taken for the internal concentration to equal the external can be assessed. The points at which equilibrium is reached for the different external con-

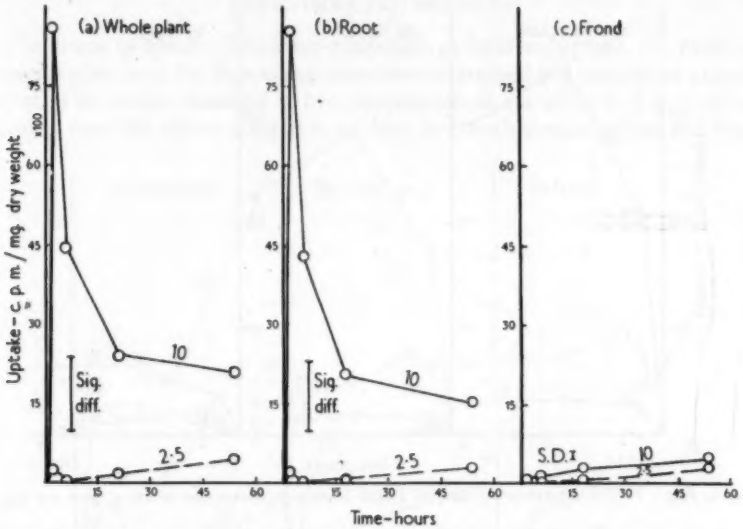


FIG. 3. *Expt. 3.* The course of uptake of TIBA from concentrations of 10 and 2·5 mg./l. The significant differences shown in this and subsequent figures are between any two treatments at $P = 0{\cdot}05$.

centrations are indicated by arrows in Figs. 1 and 2. It is to be noted that the times required are much shorter for the roots than for the fronds.

So far in discussing the changes in the root and the frond no account has been taken of their possible dependence on transport in either direction of TIBA or some iodine-labelled derivative. To investigate whether transport is an important factor a comparison was made between the uptake by intact plants and the respective uptakes by both excised roots and fronds from which the roots had been severed. Since absorption through the cut surfaces might be aberrant, in an additional series the surfaces were covered with petroleum jelly. This operation could be accomplished more readily with the fronds than with the roots and it is doubtful whether for the roots all the ends were effectively sealed.

The results given in Fig. 4 suggest that uptake by the frond and uptake by the root are largely independent processes. For the frond, although there is

some variation with time, the difference in the mean uptake over the 4·3 hours between intact fronds and those lacking roots but greased is not significant. For the root there is a significant inter-action between time and treatment. After 40 minutes' uptake by excised and intact roots is of the same order but subsequently the amount present in the intact roots is greater. The magnitude of the differences are too great to be ascribed to movement from the frond to the root. It seems more reasonable to suppose that in the severed roots depletion of carbon substrates affected the course of uptake.

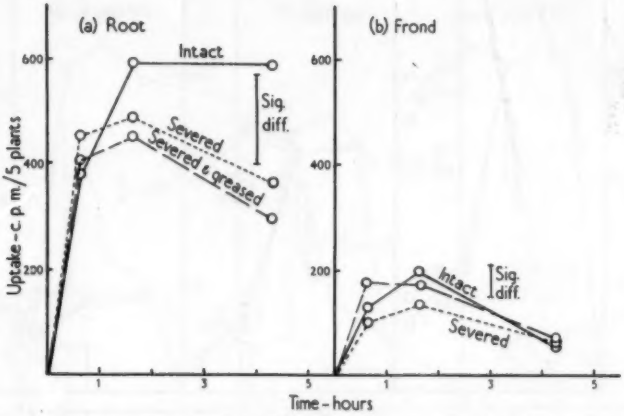


FIG. 4. *Expt. 4.* The course of uptake of TIBA from a concentration of 10 mg./l. by either the root and frond of intact plants or by roots and fronds which have been (i) severed, (ii) severed and the cut surfaces greased with petroleum jelly.

Factors determining the course of uptake

In the previous investigation (Blackman *et al.*, 1959) it was demonstrated that the course of uptake by *L. minor* of 2:4-D followed a similar pattern to that now described for TIBA. It was further shown that if plants pre-treated for 30 minutes with labelled 2:4-D were transferred either to culture solution or to culture solutions containing unlabelled 2:4-D then there was a rapid outward movement into the solution while the rate of egress was reduced by the presence of unlabelled compound in the external medium. In view of these results it seemed desirable to undertake similar experiments with TIBA.

Fig. 5 gives the results of one experiment in which plants after being treated for 2·5 hours with 10 mg./l. were placed either in culture solution or culture solutions to which 20 and 40 mg./l. of unlabelled compound had been added. As with 2:4-D, after transference there is within the first hour a rapid loss from the tissues and this is more pronounced for the root than for the frond. On the other hand, the rate of loss is not significantly affected by the external concentration.

In another experiment the initial rate of loss was studied in more detail

by making observations at shorter intervals. After pre-treatment for 2 hours with 10 mg./l. the plants were transferred to culture solution and from Fig. 6 it is apparent that even after 20 minutes the contents of the root and frond have diminished by 59 and 40 per cent.

In the detailed analysis of the loss of 2:4-D in the post-treatment period it was shown that the fall in radioactivity of the tissues could not be ascribed to the release of $^{14}CO_2$ since the loss was matched by an equivalent gain of radioactivity in the external solution. By chromatographic techniques it was

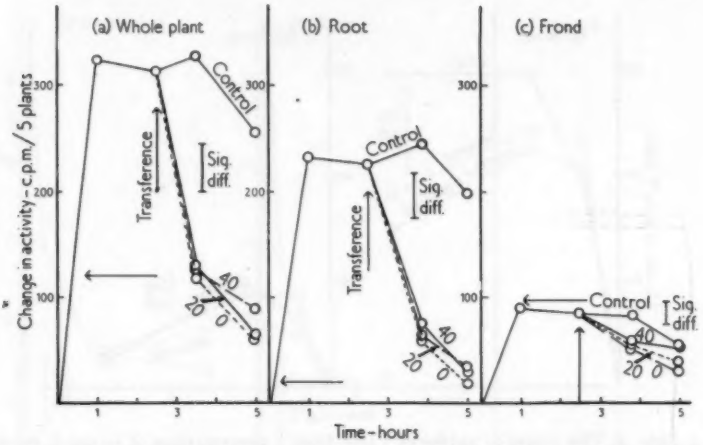


FIG. 5. *Expt. 5.* The losses of TIBA to the external solution following on the transference of plants treated with labelled compound (10 mg./l. for 2·5 hours) to solutions containing 0, 20, and 40 mg./l. of unlabelled TIBA.

established that the only radioactive compound released to the solution was 2:4-D.

In the case of TIBA it seemed highly improbable that the loss could be ascribed to the release of volatile derivatives and in consequence the only question remaining was the nature of the compound or compounds which moved out into the solution. Accordingly fronds were first treated for 2 hours with 10 mg./l. and then transferred to distilled water. After 24 hours the plants were removed, an aliquot of the solution concentrated and spotted on to chromatographic paper and run in the *iso*propanol, water, ammonia mixture. When the dried chromatographic strip was placed in the electronic scanning device, there was a major peak between Rf 0·6 and 0·7 and a very minor peak at 0·5. Since the chromatogram for the original material showed a similar peak at Rf 0·6–0·7 and a small peak at Rf 0·5 (due to traces of radioactive iodide as an impurity) it can be concluded that it is largely TIBA itself which moves out into the external solution.

On the basis of the results so far cited it can be advanced that the course of uptake has two components. At a given time the rate of uptake by the whole

plant is dependent upon the net difference between the levels of entry and egress into the roots and the fronds. Initially these rates of entry will exceed the rates of egress and the plants will accumulate TIBA. Subsequently, the rates of egress become comparatively greater until ultimately, except from very low external concentrations, they exceed the rates of entry and net uptake by the whole plant is now negative.

Inspection of Figs. 2–4 suggests that the rates of entry and egress balance after the plants have been treated for 2 to 3 hours. It would therefore be

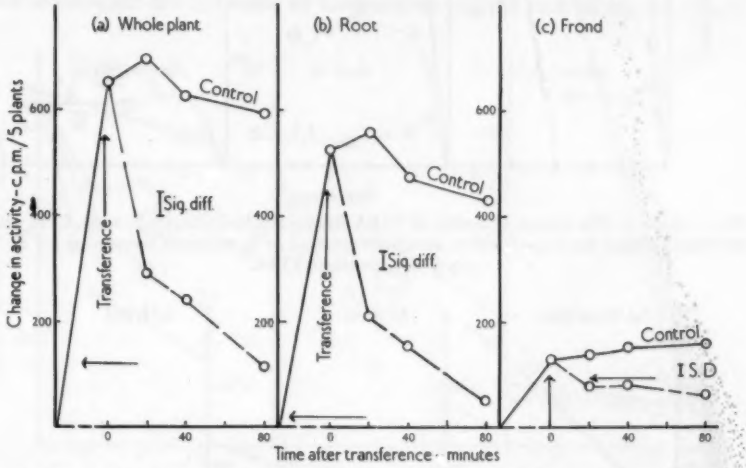


FIG. 6. *Expt. 6.* The loss of TIBA to the external solution following on the transference of plants treated with labelled compound (10 mg./l. for 2 hours) to culture solution.

anticipated that if plants are placed for 2 hours in culture solution containing 2·5 and 10 mg./l. of unlabelled TIBA and are then transferred to a solution containing 10 mg./l. of labelled compound the rates of uptake compared to those of plants receiving no pre-treatment should be markedly depressed. This expectation is confirmed by the results given in Fig. 7. What is unexpected is the relationship between the concentration during pre-treatment and the subsequent reduction in uptake. For both the whole plant and the root the rate of accumulation of the labelled compound is depressed more by pre-treatment with 2·5 as against 10 mg./l. This inverse relationship was confirmed in a second experiment.

The effect of concentration in the initial period on the subsequent uptake by the frond (Fig. 7*c*) is much smaller. It should also be noted that pre-treatment reduces the content significantly after 1 hour but not after 5 hours.

Influence of temperature on rates of entry and egress

If the inward and outward movement are taking place simultaneously any analysis of the effects of temperature is hampered by the difficulty of separat-

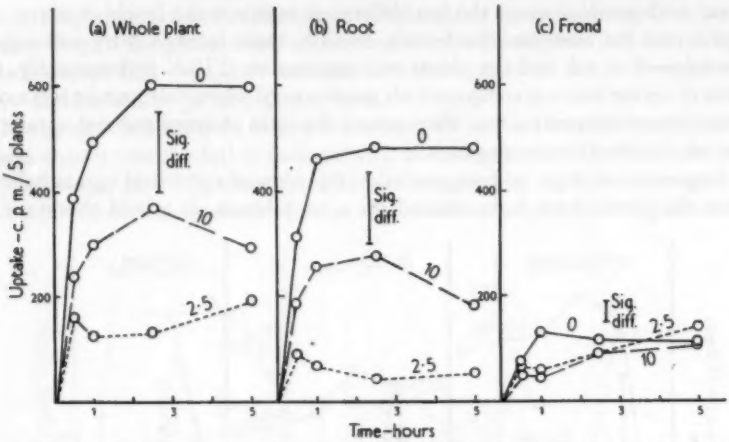


FIG. 7. *Expt.* 7. The course of uptake of TIBA from a labelled solution (10 mg./l.) of plants previously placed for 2 hours either in culture solution or in solutions containing 2·5 and 10 mg./l. of unlabelled TIBA.

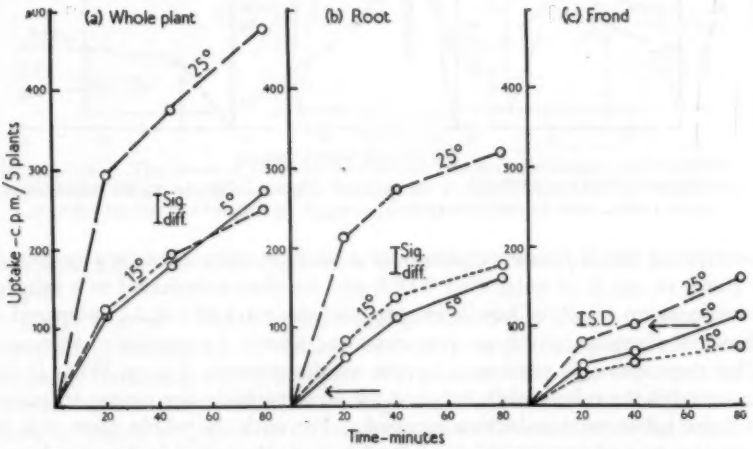


FIG. 8. *Expt.* 8. The influence of temperature on the uptake of TIBA from a concentration of 10 mg./l.

ing the two components. It has been deduced that the rate of egress compared to the rate of entry is minimal in the initial phase of absorption and therefore in studying the effects of temperature on uptake from a concentration of 10 mg./l. measurements were made at short intervals.

The results, set out in Fig. 8 have unusual features. For the first 20 minutes when interference from outward movement should be least there are no significant differences between 5 and 15° C. in the amounts found in the whole

plant, root, or frond. On the other hand between 15 and 25° C. accumulation by the root and frond is markedly accelerated. Assuming that the amounts observed after 20 minutes reflect the mean rate of uptake over the period then the temperature coefficients between 15 and 25° C. for whole plant, root, and frond are 2·36, 2·56, and 1·98 respectively. Between 20 and 40 minutes the further amounts accumulating in the root and frond are no more marked at 25° C. than at 15° C. and in consequence there is no longer a major temperature effect.

To investigate the influence of temperature on the rate of egress, plants

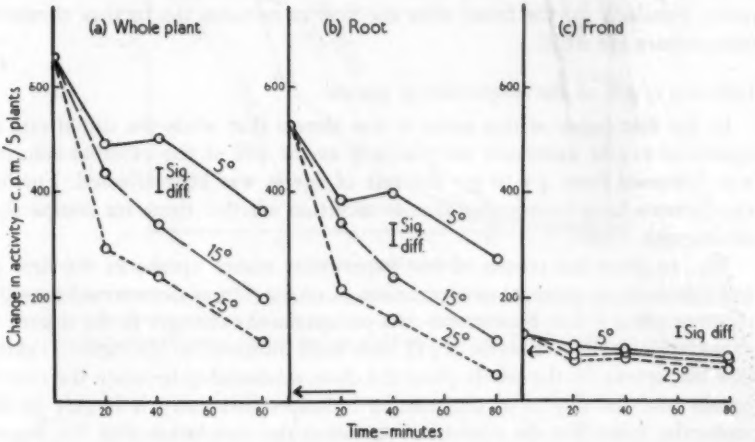


FIG. 9. *Expt. 9.* The effect of temperature on the initial rates of loss to culture solution of plants previously treated with labelled TIBA (10 mg./l.) for 2 hours.

were placed in 10 mg./l. of unlabelled TIBA for 2 hours at 25° C. and then transferred to culture solution maintained at 5, 15, and 25° C. and the amounts lost from the plants determined after 20, 40, and 80 minutes. Comparing Figs. 8 and 9 it is apparent that the effects of temperature on egress are different from those on entry since raising the temperature from 5 to 15° C. has a considerable influence over the whole 80 minutes on the rate of loss from both the whole plant and the root. In the first 20 minutes the temperature coefficients between 5 and 15° C. for the losses from the whole plants, roots, and fronds are 1·34, 1·29, and 1·66 while the corresponding figures for the interval 15° to 25° C. are 1·64, 1·70, and 1·37. It is also of interest that the proportion lost by the root is greater. On a percentage basis the reduction at 5, 10, and 15° C. are 27, 35, and 59 for the root and 18, 29, and 40 for the frond.

The interpretation of the effects of temperature after 20 minutes presents some difficulty since the amounts remaining in the root and frond at that time are different for the three temperatures and it is likely that the outward

transference will be dependent on both the temperature and the initial internal content. The fact that between 20 and 40 minutes the content of the root tends to go up at 5° C. and down at 15° C. (the interaction is highly significant) indicates that the rate of egress is still temperature dependent. In contrast over the same period the loss from the root at 15° C. exceeds that at 25° C. but at 15° C. the content at the start is higher. Again, between 40 and 80 minutes the losses and the initial concentrations at 5 and 15° C. are both somewhat greater than those at 25° C. Thus although it is not possible to separate precisely the effects of temperature and concentration the general trends suggest that the temperature coefficients cannot depart much from unity. Similarly for the frond after the first 20 minutes the further effects of temperature are small.

Influence of pH on the components of uptake

In the first paper of this series it was shown that while the initial rate of uptake of 2:4-D decreased curvilinearly as the pH of the external solution was increased from 4·1 to 5·1 the rate of egress was little affected. Further experiments have been undertaken to ascertain whether there are comparable effects with TIBA.

Fig. 10 gives the results of one experiment where uptake in the first 30 minutes from an external concentration of 10 mg./l. was determined over the range of pH 4·1–6·1. Besides the data on uptake the changes in the degree of dissociation at the five levels of pH have been indicated in the figure. Taking first the uptake by the whole plant the close relationship between the rate of uptake and the degree of dissociation indicates that entry is largely in the molecular form. For the content of the frond the correlation with the degree of dissociation is greater than for the root. The major discrepancy for the roots is their failure to absorb more when the pH changes from 4·6 to 4·1. Previous experiments (Blackman and Robertson-Cunninghame, 1953) have shown that the optimal pH for the growth of *L. minor* in the same culture solution is 5·1 and it is possible that at pH 4·1 there is some injurious effect.

In the corresponding experiment on the changes induced in the outward movement the plants were first placed in a solution containing 10 mg./l. at a pH of 5·1 for 2 hours transferred to culture solutions adjusted to five different hydrogen-ion concentrations and the amounts lost after 1 hour estimated. From Fig. 11 it is apparent that between pH 4·6 and 6·1 there are no significant differences for the losses of the whole plants, roots, or fronds. Below pH 4·6 the rate for the frond remains unchanged but the rate for the root and thereby the rate for the whole plant is reduced. It could be advanced that this diminution is also brought about by the injurious effects of the low pH.

Influence of 2:3:5-triiodobenzoic acid on the uptake of Cerium-144

The evidence so far presented is consistent with the view that entry and egress of TIBA is in the molecular form and that within 2–3 hours of the plants being placed in a concentration of 10 mg./l. at pH 5·1 the physiological

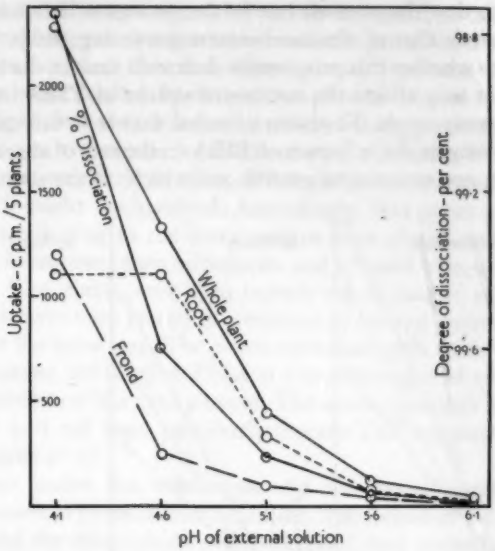


FIG. 10. *Expt. 10.* Uptake of TIBA in the first 30 minutes from a concentration of 10 mg./l. in relation to the hydrogen-ion concentration of the external solution. For purposes of comparison the variation in the degree of dissociation of TIBA is also shown.

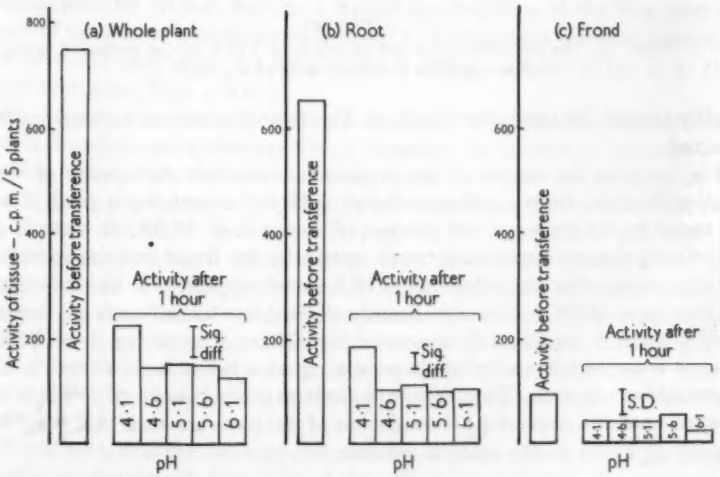


FIG. 11. *Expt. 11.* The reductions in content of TIBA in the first hour following on the transference of plants treated with 10 mg./l. (pH 5·1) for 2 hours to culture solution adjusted to different concentrations of hydrogen ion.

effects are such that the over-all rate of the processes determining outward movement exceeds that of the mechanisms governing entry. The question then arises as to whether this progressive shift with time in the balance of two components not only affects the net rate of uptake of TIBA but also that of other types of compounds. To obtain a limited answer to this query it seemed simplest to investigate the influence of TIBA on the rate of absorption of some cation which is not essential to growth and which, at the same time, can be

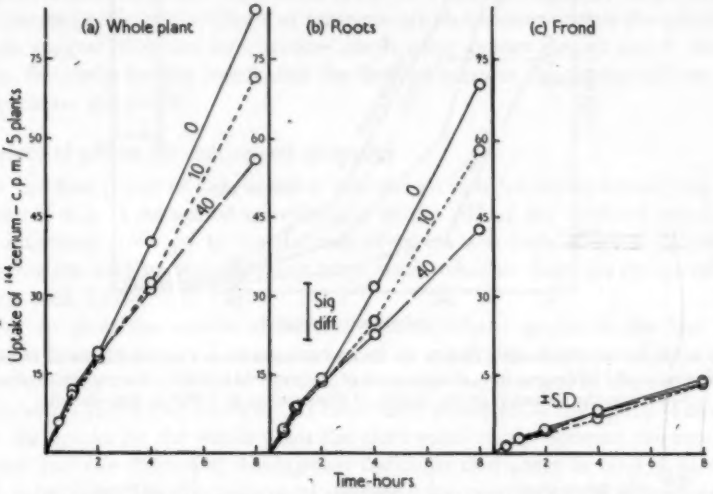


FIG. 12. *Expt. 12.* The influence of 10 and 40 mg./l. of TIBA on the course of uptake of cerium-144 from a concentration of 0·5 mg./l.

readily assayed by isotopic techniques. On these two counts cerium-144 was selected.

Fig. 12 gives the results of one experiment in which the uptake of ^{144}Ce (as the chloride) from a culture solution (pH 5·1) containing 0·5 mg./l. was measured in the presence and absence of 10 mg./l. of TIBA. In view of the differences already observed between uptake by the frond and the roots it is not surprising that the effects of TIBA are divergent. For the frond the accumulation of ^{144}Ce is not significantly affected but for the roots the content after 8 hours is significantly depressed by either 10 or 40 mg./l. of TIBA. It is of some significance that there is a lag of 2 hours before there is any appreciable reduction. Thus it would seem that the uptake of ^{144}Ce is not affected until the physiological conditions of the roots are such that there is a net loss of TIBA to the external solution.

The effects of prior freezing on uptake of 2:3:5-triiodobenzoic acid

It has been demonstrated that when the temperature is lowered from 15 to 5° C. (Fig. 8) for the first 20 minutes there is no significant reduction in the

rate of uptake of TIBA by either the frond or the root. This lack of a temperature effect would suggest that for both tissues the processes controlling uptake are physical rather than chemical, that is to say processes of diffusion and adsorption on suitable sites. It seemed therefore of interest to investigate the level of uptake at *circa* 0° C. Further consideration suggested that it might also be illuminating to investigate the effects of a prior freezing of the plants since such a treatment was likely to affect differentially any metabolic and physical systems which might be involved. Accordingly, thin pyrex glass tubes containing plants adhering to the inner surface were placed in liquid air for 2 minutes, the tubes were then withdrawn and allowed to equilibrate to room temperature. The plants were then transferred to culture solution in small beakers which were then placed in a mixture of ice and water for 1 hour and illuminated in the usual way. The plants were then again transferred to similar beakers containing the labelled TIBA at a concentration of 10 mg./l. and the uptake measured over the next 5 hours. The same procedure was adopted for plants which had not been previously frozen. The temperature inside the beakers averaged 1° C.

Examination under the microscope did not reveal significant signs of damage as a result of immersion in liquid air. The tissues of the frond and root were intact and the chloroplasts still maintained their normal shape.

It is clear from Fig. 13 that subjecting the control plants to a temperature of 1° C. does not inhibit the accumulation of TIBA by either the root or the frond, in fact comparison with Fig. 8 shows that after 30 minutes the contents of TIBA in the fronds and roots at 1° C. and 5° C. are of the same order. Another feature is that there is a steady accumulation in the root over the whole of the 5-hour period, whereas at 25° C. for the same external concentration of TIBA after *circa* 2–3 hours there is either a net loss (Figs. 2, 3, 5) or no further gain (Figs. 4 and 7).

Turning to the plants first frozen in liquid air, between the roots and fronds the effects on uptake are widely disparate. In the first 30 minutes prior freezing diminishes the content of the roots by 66 per cent. while in the fronds the content is augmented by 172 per cent. This greater accumulation of the 'frozen' fronds is maintained throughout the experiment and after 3·5 hours the content is 3·6 times as great as in the normal fronds, due in part to the induced delay in the time taken before the phase of accumulation is replaced by the phase of outward movement. Whereas at 30 minutes the pre-treated roots contained less TIBA, at 2 hours they had more than caught up with the controls, but between 2 and 3·5 hours there was a sharp reversal since while the control roots continued to gain TIBA, the pre-frozen roots lost it.

It is known that the freezing and thawing of plant tissues although usually causing much internal disruption of the cells does not lead to the cessation of all metabolic processes. It seemed therefore of interest to carry out another experiment in which the course of uptake of either control plants or plants treated with liquid air was followed at 25° C. On the basis of the experiments

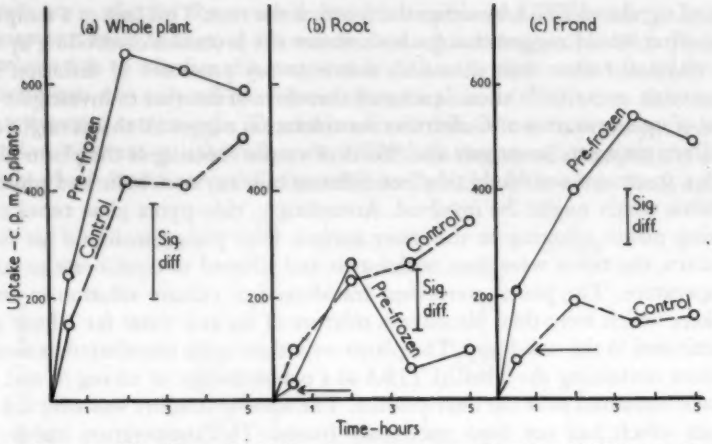


FIG. 13. *Expt. 13.* The effects of prior freezing on the course of uptake of TIBA at 1° C. from a concentration of 10 mg./l.

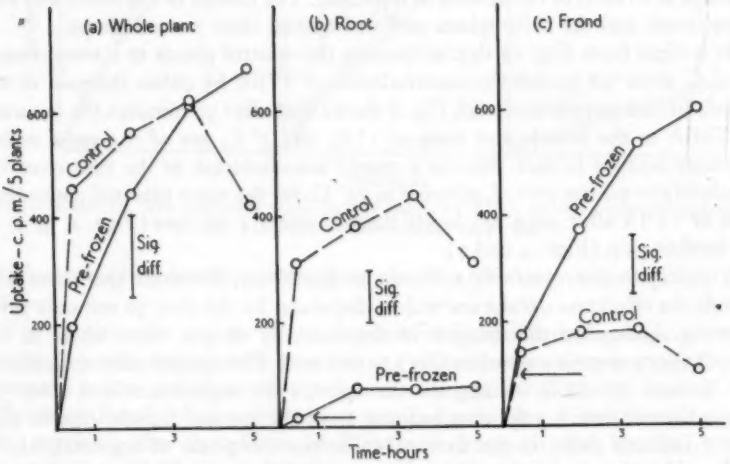


FIG. 14. *Expt. 14.* The effects of prior freezing on the course of uptake of TIBA at 25° C. from a concentration of 10 mg./l.

already described it would be expected that the initial rates of accumulation by the roots and frond of normal plants would be greater at 25° C. than at 1° C. and a comparison of Figs. 13 and 14 shows that in the first 30 minutes this expectation is confirmed. Further inspection of the two figures shows that for the pre-frozen roots and fronds there is no corresponding temperature effect since the rates of uptake at 1° and 25° C. are of the same order. In consequence, at 25° C. as compared to 1° C., after 30 minutes the relative reduc-

tion in the content of the roots induced by prior freezing is increased. In contrast the content of the fronds at 25° C. is not statistically below that of the controls, but it is at 1° C. (Figs. 13 and 14).

Between 0·5 and 3·5 hours the rate of accumulation of TIBA by the frozen frond at 25° C. follows that at 1° C., and once more greatly exceeds the gains of the control fronds. A further comparison of Figs. 13 and 14 reveals that the behaviour of the frozen roots is different at the two temperatures; the roots at 25° C. unlike those at 1° C. do not show a marked fall in content.

The influence of indole-3-acetic acid on the uptake of 2:3:5-triiodobenzoic acid

As previously stated in the introduction there is evidence that TIBA can interfere with some of the physiological actions of indole-3-acetic acid (IAA)

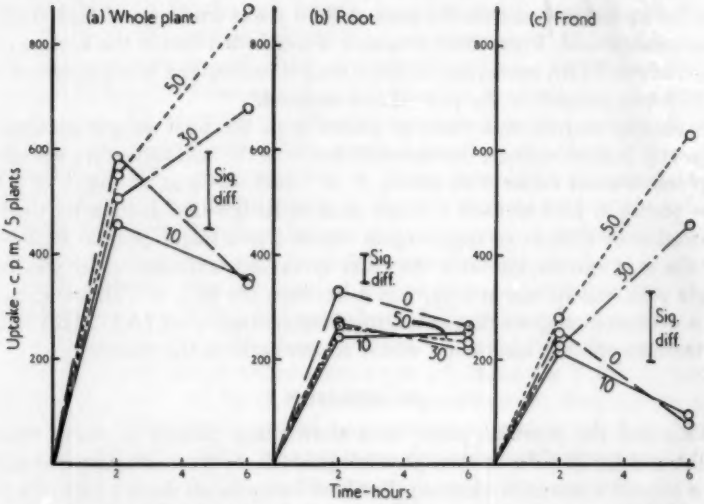


FIG. 15. *Expt. 15.* The interrelationship between the external concentration of IAA (0, 10, 30, and 50 mg./l.) and the course of uptake of TIBA from a concentration of 10 mg./l.

and it seemed therefore relevant to investigate the influence of IAA on the uptake of TIBA. The concentration of labelled TIBA in the culture solution (pH 5·1) was kept constant at 10 mg./l. while the concentration range of IAA was 0, 10, 30, and 50 mg./l. The results—see Fig. 15—demonstrate that for the frond and the root the effects of IAA are widely divergent. For the frond, adding 10 mg./l. of IAA does not alter the change in content with time, but when the concentrations are increased to 30 and 50 mg./l. instead of the content falling after 2 hours it continues to rise, so that at the end of 6 hours the fronds subjected to the highest concentration contain 8·2 as much as the controls.

In contrast, the changes induced by IAA in the root are relatively small.

At the end of 2 hours IAA over all concentrations has reduced the content of TIBA by 21 per cent. but at 6 hours the differences from the controls are slight.

The fate of triiodobenzoic acid after absorption

The possible conversion of TIBA after absorption into other compounds was examined by the chromatographic procedures already described (p. 483). In one experiment batches of plants were placed in 10 mg./l. of TIBA and sampled at 2·5 and 24 hours. For both sampling occasions the ether re-extract exhibited a much higher activity than the acid extract. At 2·5 hours the trace on the scanner for both extracts showed a major peak between Rf 0·6 and 0·7 (TIBA), a minor peak at 0·5 (iodide), and indications of activity at Rf 0·9 in the ether re-extract. The same general trend held for the plants left in the solution for 24 hours save that the peak at Rf 0·5 was smaller and that at Rf 0·9 more pronounced. From these results it is concluded that in the first 24 hours much of the TIBA remaining in the tissues is unchanged or is converted back to TIBA as a result of the procedures adopted.

A similar analysis was made of plants from the final sample of Expt. 15 (Fig. 15). In this instance, the chromatograms of the acid and either re-extracts of plants treated either with 10 mg./l. of TIBA alone or 10 mg./l. of TIBA plus 30 mg./l. IAA showed a single peak at Rf 0·6–0·7. Increasing the concentration of IAA to 50 mg./l. again resulted in a major peak at Rf 0·6–0·7 for the acid extract, but with the ether re-extract, although there was but a single peak, the Rf was at 0·55, that is between the Rf's of TIBA and iodide. This evidence suggests that at a critical concentration of IAA TIBA is converted into another compound which accumulates in the tissues.

DISCUSSION

This and the previous paper have shown that though in many respects TIBA and 2,4-D differ in their physiological effects the mechanisms of uptake have several features in common. For both compounds the net rate of uptake by whole plants of *L. minor* is dependent on the differences between the processes or mechanisms governing the rates of entry and egress. Initially the rate of entry exceeds the rate of egress, but by the end of 1–4 hours these two rates reach equilibrium and the net uptake ceases. Subsequently the rate of egress may exceed the rate of entry, but whether the net uptake of the whole plant does assume a negative value is dependent on the external concentration. For TIBA inspection of Figs. 1 and 2 suggests that the net loss is not induced unless the concentration exceeds 2·5 mg./l.

Although it has already been emphasized that the results of Fig. 4 are not conclusive they provide strong evidence that the changes in content observed in the root and frond are due to direct entry or egress rather than to movement from one organ to the other. The fact that in many experiments the trends for roots and fronds are so disparate lends further support to the view that transport in either direction is not of major significance. For instance, there can be

marked gains or losses in the content of the roots without any corresponding or compensatory changes in the content of the frond, e.g. Figs. 3, 5, 7, 9, and 10. It therefore seems legitimate to consider the trends for the roots and frond separately especially when the time interval is short.

Taking first the initial phase of entry, that is entry during the first 30 minutes, it has been concluded that entry into both the roots and frond is largely as undissociated molecules (p. 492) and in this respect TIBA is similar to 2,4-D when taken up by *L. minor* (Blackman *et al.*, 1959) or by Chlorella (Wedding and Erickson, 1957). In contrast Johnson and Bonner (1956) found that the influence of the external pH and the concomitant changes in the degree of dissociation had a much smaller influence on the uptake of 2,4-D by *Avena* coleoptiles while similar results for the uptake of IAA by carrot disks and segments of pea stem were obtained by Reinhold (1954) and van de Westeringh (1957). Bennett (1955), working with Nitella, determined at different levels of pH the concentrations of IAA and 2-methyl-4-chlorophenoxyacetic acid which stopped protoplasmic streaming. He concluded that if only undissociated molecules were involved then the relationship between pH and the equi-effective concentration was satisfied if it was assumed that at the surface of the external interface the pH was shifted 1·1 below the pH of the external solution. Thus, it could be advanced that the specific differences recorded might in part be ascribed to a varying shift in the pH at the outer interface, but on general grounds it seems unlikely that the nature of the surface would be so different as to allow of so large a variation.

Considering next the influence of temperature on uptake in the first 20 minutes the results of Fig. 8 diverge from those previously observed for the initial absorption of 2,4-D where between 7·5 and 30° C. the temperature quotient for whole plants varied from 2·3 to 2·6. Since for TIBA the rates of uptake by the root and frond show little dependence on temperature up to 15° C. and coefficients of 2·5 and 1·95 between 15 and 25° C. it is simplest to suppose that up to 15° C. the processes determining absorption are largely physico-chemical and that as the temperature further increases metabolic processes come much more into play. This supposition implies that the influence of temperature on the over-all metabolic rate is unusual in that below 15° C. the rate is very low. In this connexion it is of interest to note that an adenyl pyrophosphatase preparation from potatoes only hydrolysed both labile groups of ATP above 7° C. (Lee and Eiler, 1951).

Turning to the processes governing the rate of egress from *L. minor* some of the characteristics exhibited by TIBA are similar to those of 2:4-D (Blackman *et al.*, 1959) while others are divergent. For both compounds the magnitude of the temperature coefficients for the losses from whole plants in the initial period after transference are of the same order. For TIBA, where the losses from the root and frond have been estimated separately, it can be concluded that since the temperature coefficients range from 1·29 to 1·70 (see p. 491) physico-chemical processes play an important part if not a dominant role. Another characteristic in common is that the rate of egress is little

affected by the external pH. On the other hand the effect of the external concentration of unlabelled compound on the losses to the external medium are divergent. As the external concentration of 2,4-D is increased the rate of egress is decreased, while for TIBA it is apparent from Fig. 5 that the rate for either the roots or frond is not linked with the external concentration. In neither case can the mechanism be one of 'exchange' since the rate of egress would be augmented by the presence of the unlabelled compound in the solution. Nor can it be a question of diffusion from free space because the amounts lost are far too great. For example, inspection of Fig. 6 shows that in the first 20 minutes the losses from root equalled seventeen times the amount which would be found in the tissues if the mean internal concentration equalled the external. For the frond the corresponding figure is much less, namely 50 per cent., but this is nevertheless higher than would be expected for diffusion from free space.

Comparing further the mean internal and external concentrations the greatest recorded differences for both roots and fronds are at pH 4·1 (Fig. 10), where the internal concentrations exceed the external (10 mg./l.) by a factor of circa 50 and 13 respectively. In several other experiments the corresponding factor for the roots ranged from 22 to 29 (Figs. 2, 4, and 7, 11), while the equivalent for the frond was only 1 to 2.

Further investigation is demanded before it can be shown with precision what are the sites of accumulation in the tissues, that is to say whether accumulation is confined to the cytoplasm (including components of the cell wall) or whether the vacuole is involved. When autoradiographs were made of treated plants they showed that accumulation was greatest in the root tip, and this was confirmed in an experiment where the roots were divided into three equal parts and the segments counted separately. After exposure to 10 mg./l. for 4·3 hours the radioactivity of the tip, middle, and basal sections was in the proportion of 4:2:1. This preferential accumulation in the root tip suggests a linkage with a high metabolic rate but not necessarily accumulation in the vacuole.

If accumulation is confined to the cytoplasm it seems that any binding or chemical attachments which do occur must be weak or readily reversible, since otherwise it would not be expected that after transference such a large proportion would be quickly released to the external solution (Figs. 5 and 6). In fact it could be advanced that such a rapid loss is not compatible with accumulation in the vacuole.

Leopold and Price (1957) have shown that *in vitro* TIBA reacts with sulphydryl groups and could in consequence interfere with the activity of co-enzyme A. If it is assumed that *in vivo* only a small proportion of the contained TIBA is involved in this or similar types of reactions then the physiological disturbance could still be cumulative and bring about, for example, the reversal from a positive to a negative uptake. It should, however, be emphasized that this physiological shift can hardly be regarded as a toxic effect since Sargent and Wangermann (1959), working at a somewhat lower pH

(4·8), have found that the induced increase in venation did not attain a maximal level until the external concentration of TIBA was 10 mg./l. and the duration of treatment 3 days.

It has been shown (Figs. 13 and 14) that prior freezing results in a decreased uptake by the root but a greater absorption by the frond. As the plants exhibited little visible injury it was decided to assess the degree of damage by measuring in a Warburg manometer the oxygen consumption at 25° C. of normal and pre-frozen plants. When observations were made an hour after freezing then the oxygen uptake of treated plants was half that of control plants, but if the pre-frozen plants were returned to the illuminated water bath then after 5 hours the respiration had almost stopped.

These findings taken in conjunction with the facts that the rates of accumulation in the pre-frozen frond were of the same order at 25° and 1° C. and that uptake continued over the 5 hours led to the conclusion that the additional uptake over the control fronds was dependent on physico-chemical processes and that the disruption caused by freezing increased the number of reactive sites or surfaces or facilitates access to them. If this assumption is correct the question arises as to why freezing depressed root uptake, and it can only be advanced that any extra absorption due to additional reactive centres must be more than offset by losses arising from interference in the normal mechanisms of absorption. Thus, if accumulation takes place largely in the vacuole, cytoplasmic injury could prevent this. It should also be borne in mind that the total volume of tissue in the frond is greater than in the root and that freezing might on a *pro rata* basis liberate more sites. Moreover, since uptake by the normal frond is far less than the root it may be that accumulation is localized in the cells adjacent to the under-surface and that as a consequence of cellular disorganization due to freezing penetration throughout the tissues takes place.

In this connexion it is of some significance that high concentrations of IAA like freezing increase uptake by the frond (Fig. 15). In other experiments it was found that such concentrations (30 and 50 mg./l.) cause severe epinasty, and these effects might well be preceded by internal physiological changes, leading, like freezing, to additional and possibly different active sites. As a corollary it must be advanced that the roots are more physiologically tolerant of high external concentrations of IAA and that in consequence uptake of TIBA is relatively unaffected.

The considerations put forward to account for the effects of freezing and IAA can also offer some explanation of the changing pattern of uptake. It would seem that initially uptake is not limited by the availability of sites or paths of active transport, but as the content of TIBA rises the physiological conditions are altered so that the number or character of the sites are changed and active transport is reduced. In addition, any accumulation in the vacuole will be retarded, stopped, or there may be a reverse flow through the tonoplast and the cytoplasm if the barriers to outward movement from the vacuole are altered. At the present state of knowledge it is not possible to identify the

mechanisms involved. Sargent and Wangermann (1959), examining the interaction of TIBA and IAA on venation, have concluded that the changes induced by TIBA could not be ascribed to an indirect effect of TIBA suppressing the content of endogenous IAA. Nor can their results be explained on the basis that TIBA interferes with the polar transport of IAA which has been demonstrated in other species by Hay (1956), Niedergang-Kamien and Skoog (1956), and Zwar and Rijven (1956). More recently Kessler and Moscicki (1958) have shown that subsequent to foliar application of TIBA the movement of calcium and iron applied to the leaves was facilitated but no distinction can be made between the effects of TIBA on penetration and transport. Thus, the present postulation that TIBA may interfere with transport mechanisms within the cell is not incompatible with the findings of previous workers. On the other hand it must be stressed again that although TIBA in many respects differs from 2,4-D yet the general pattern of uptake by *L. minor* is similar and it may be that the essential features these compounds share are their auxin-like properties which determine the pattern of uptake, properties which, from a fundamental point of view, still remain ill defined or unknown.

LITERATURE CITED

Åberg, B. (1950). On auxin antagonists and synergists in root growth. *Physiol. Plantarum*, **3**, 447.

—— (1953). On the interaction of 2,3,5-triiodobenzoic acid and maleic hydrazide with auxins. Ibid. **6**, 277.

—— (1957). Auxin relationships in roots. *Ann. Rev. Plant Physiol.* **8**, 153.

Applegate, H. G., and Hamner, C. L. (1957). Effect of foliar application of substituted benzoic acids on leaf development in *Zinnia elegans*. *New Phytol.* **56**, 301.

Audus, L. J., and Thresh, R. (1956). The effects of synthetic growth-regulator treatments on the levels of free endogenous growth substances in plants. *Ann. Bot.* N.S. **20**, 439.

Bennett, M. C. (1955). The influence of cell membrane on some toxicity effects of growth substances in Nitella. *J. Exp. Bot.* **6**, 276.

Blackman, G. E., and Robertson-Cunninghame, R. C. (1953). The influence of pH on the phytotoxicity of 2,4-dichlorophenoxyacetic acid to *Lemna minor*. *New Phytol.* **52**, 71.

——, Sen, G., Birch, W. R., and Powell, R. G. (1959). The Uptake of Auxins. I. Factors controlling the uptake of phenoxyacetic acids by *Lemna minor*. *J. Exp. Bot.* **10**, 33.

Bonner, J. (1949). Further experiments on flowering in Xanthium. *Bot. Gaz.* **110**, 625.

De Waard, J., and Floroschutz, P. A. (1948). On the interaction of 2,3,5-triiodobenzoic acid and indole-3-acetic acid in growth processes. *Proc. Kon. Akad. Wetensch. Amsterdam*, **51**, 1317.

—— and Roodenburg, J. W. M. (1948). Premature flower bud initiation in tomato seedlings caused by 2,3,5-triiodobenzoic acid. Ibid. **51**, 3248.

Galston, A. N. (1947). Effect of 2,3,5-triiodobenzoic acid on the growth and flowering of soyabeans. *Amer. J. Bot.* **35**, 356.

Gorter, C. J. (1954). The flowering response of *Phaseolus vulgaris* to 2,3,5-triiodobenzoic acid and its Cl- and Br- analogues. *Proc. Kon. Akad. Wetensch.* Series C. **57**, 606.

Hay, J. H. (1956). The effect of 2,4-dichlorophenoxyacetic acid and 2,3,5-triiodobenzoic acid on the transport of indole-acetic acid. *Plant Physiol.* **31**, 118.

Harder, R., and Oppermann, A. (1952). Einfluss von 2,3,5-triiodobenzo esaure auf die Blüten bildung und die vegetative Gestaltung von *Kalanchoë Blossfeldiana*. *Planta*, **41**, 1.

Johnson, M. P., and Bonner, J. (1956). The uptake of auxin by plant tissue. *Physiol. Plantarum*, **9**, 102.

Kessler, B., and Moscicki, Z. W. (1958). Effect of triiodobenzoic acid and maleic hydrazide upon the transport of foliar applied calcium and iron. *Plant Physiol.* **33**, 70.

KRAUS, E. J., BROWN, N. A., and HAMNER, K. C. (1936). Histological reaction of bean plants to indoleacetic acid. *Bot. Gaz.* **98**, 370.

LEE, KWAN-HUA, and EILER, J. J. (1951). Temperature dependent characteristics of an adenyl pyrophosphatase preparation from potatoes. *Science*, **114**, 393.

LEOPOLD, A. C., and PRICE, C. A. (1957). Sulfhydrils in plants. I. Reactions with growth regulators. *Plant Physiol.* **32**, 500.

LOUGHMAN, B. C., and MARTIN, R. P. (1955). Methods and equipment for the study of the incorporation of phosphorus in intact barley plants in experiments of short duration. *J. Exp. Bot.* **5**, 272.

MUIR, R. M., and HANSCH (1951). The relationship of structure and plant growth activity of substituted benzoic and phenoxy acetic acids. *Plant Physiol.* **26**, 269.

NIEDERGANG-KAMIEN, E., and SKOOG, F. (1956). Studies on polarity and auxin transport in plants. I. Modification of polarity and auxin transport by triiodobenzoic acid. *Physiol. Plantarum*, **9**, 60.

REINHOLD, L. (1954). The uptake of indole-3-acetic acid by pea epicotyl segments and carrot discs. *New Phytol.* **53**, 217.

SARGENT, J. A., and WANGERMANN, E. (1959). The effect of some growth regulators on the vascular system of *Lemna minor*. *New Phytol.* In press.

SNYDER, W. E. (1949). Some responses of plant to 2,3,5-triiodobenzoic acid. *Plant Physiol.* **24**, 295.

STEINBURG, R. A. (1941). Use of *Lemna* for nutritional studies in green plants. *J. Agric. Res.* **62**, 423.

STREET, H. E. (1955). Factors controlling meristematic activity in excised roots. VI. Effects of various 'anti-auxins' on the growth and survival of excised roots of *Lycopersicum esculentum*. *Physiol. Plantarum*, **8**, 48.

THIMANN, K. V., and BONNER, W. D. (1948). The action of triiodobenzoic acid on growth. *Plant Physiol.* **23**, 150.

VAN DE WESTERINGH, C. (1957). *Onderzoltingen over planten groeistoffen*. Doct. Thesis, Leiden Univ.

WANGERMANN, E., and LACEY, H. J. (1953). Studies in the morphogenesis of leaves. IX. Experiments on *Lemna minor* with adenine triiodobenzoic acid and ultra-violet radiation. *New Phytol.* **52**, 298.

WEDDING, R. T., and ERICKSON, L. C. (1957). The role of pH in the permeability of Chlorella to 2,4-D. *Plant Physiol.* **32**, 503.

WEINTRAUB, R. L., BROWN, J. W., NICKERSON, J. C., and TAYLOR, K. N. (1952). Studies on the relation between molecular structure and physiological activity of plant growth regulators. I. Abscission inducing activity. *Bot. Gaz.* **113**, 348.

WENCK, U. (1952). Die Wirkung von Wuchs- und Hemmstoffen auf die Blatt-form. *Zeit. für Botanik*, **40**, 33.

ZIMMERMAN, P. W., and HITCHCOCK, A. E. (1942). Flowering habit and correlation of organs modified by 2,3,5-triiodobenzoic acid. *Contrib. Boyce Thomp. Inst.* **12**, 491.

—— —— (1949). 2,3,5-triiodobenzoic acid influences flower formation of tomatoes. Ibid. **15**, 353.

ZWAR, J. A., and RIJVEN, A. H. G. C. (1956). Inhibition of transport of indole-3-acetic acid in the etiolated hypocotyl of *Phaseolus vulgaris* L. *Austral. J. of Biol. Sci.* **9**, 528.

Studies in Dormancy of Sycamore

II. THE EFFECT OF DAYLENGTH ON THE NATURAL GROWTH-INHIBITOR CONTENT OF THE SHOOT

I. D. J. PHILLIPS AND P. F. WAREING[1]

Department of Botany, University of Manchester

SUMMARY

1. An investigation was made into the effect of daylength conditions on the inhibitor content of first-year seedlings of sycamore (*Acer pseudoplatanus*).

2. The shoot apical regions and mature leaves were extracted with 80 per cent. aqueous methanol, fractionated by paper chromatography in isopropanol/ammonia and assayed by the wheat-coleoptile growth test.

3. A growth inhibitor was present in all extracts at R_f 0·7. Higher levels of inhibitor were present in both apices and mature leaves of plants transferred to short-day conditions than of those maintained under long-days throughout.

4. These differences in inhibitor level can be detected after 2–5 days of short-day treatment, *preceding* any marked effect of daylength on growth.

5. Evidence is adduced in support of the hypothesis that the inhibitor is produced in the leaves during darkness and is transported to the apex during the photoperiod.

INTRODUCTION

SEEDLINGS of sycamore (*Acer pseudoplatanus*) show marked photoperiodic responses in relation to dormancy. Under short-days (S.D.) the seedlings rapidly cease extension growth and form resting buds. Under long-days (L.D.), (e.g. 18-hour photoperiods), or continuous illumination, the period of extension growth is much greater, but the seedlings ultimately form resting-buds even under these conditions (Wareing, 1954).

It has been shown that these responses are determined primarily by the daylength conditions to which the *leaves* are exposed (independently of the photoperiodic conditions under which the shoot-apex is maintained), and it is evident that the leaves must have some inhibiting influence upon the activity of the apical meristem when maintained under S.D. It was postulated, therefore, that under S.D. the leaves of woody species must produce a growth inhibitor which prevents further extension growth by the shoot apex, and induces the formation of resting buds (Wareing, 1951, 1954).

The presence of a growth inhibitor in the shoot apex and leaves of sycamore has already been demonstrated (Phillips and Wareing, 1958), and it was shown for trees growing under natural conditions, that seasonal changes in the inhibitor content throughout the year could be correlated with the state of dormancy of the shoot apex.

This paper deals with an investigation of the changes in inhibitor content

[1] Now at University College of Wales, Aberystwyth.

Journ. of Experimental Botany, Vol. 10, No. 30, pp. 504-14, Oct. 1959.

observed when seedlings of sycamore are maintained under long-day and short-day conditions.

Extracts were prepared from the apical region and mature leaves, and were then chromatographed and assayed for biological activity with either wheat coleoptile sections or lettuce seeds.

MATERIALS AND METHODS

Plant Material. In all experiments first-year seedlings were subjected to certain photoperiodic treatments. These seedlings were collected from the open in mid March and grown in 4-inch pots in a heated greenhouse until they had attained a height of approximately 9 inches, and had fully expanded at least 2 pairs of leaves. In Expts. 1 and 2 they were then transferred directly to the treatments, but in Expts. 3 and 4 they were grown outdoors for a short period (10 days) before being brought back inside for the photoperiodic treatments.

Harvesting of the plants for extraction was done in as short a time as possible, the severed parts being deposited in a container surrounded by solid carbon dioxide. Apart from the apices only mature leaves were taken for extraction, since interest was focused upon the relationships between these and the apex under different day-length conditions.

The extracts of 'apices' were actually prepared from the shoot-tips, including the very young expanding leaves and the small non-elongated uppermost internode. The largest leaves included in apical samples were of the order of 0·5 cm. in length, none of them having opened out.

Before being extracted, the material was always kept at −15° C.

Extraction technique. The methods of extraction employed in this work were very similar to those used in previous work on the sycamore inhibitor (Phillips and Wareing, 1958).

Briefly, it involved three successive extractions at 0° C. with 80 per cent. redistilled methanol (MeOH) and concentration of this extract by distillation *in vacuo* at 35° C. followed by freeze-drying. Chlorophylls were removed from extracts by partition with 80/100 petrol ether.

Slight modifications of this technique were employed in Expts. 3 and 4, and they are described in the accounts of these.

Eighty per cent. MeOH extracts were stored at 0–2° C. prior to concentration and this period was always kept as short as possible. After reduction to the aqueous phase they were kept frozen at −15° C.

Paper-chromatography. Measured volumes of the concentrated extracts were strip-loaded on to Whatman's No. 1 paper and the chromatograms developed by the descending method, in the dark, at room temperature. The solvent was 80 parts isopropanol/20 parts aqueous ammonia (S.G. 0·88 ammonia solution × 1/100). Chromatograms were divided up for bioassay in one of two ways. Either the whole chromatogram was assayed for activity by cutting it up into transverse strips which were eluted and bioassayed, or only the region R_f 0·55–0·88 was removed, eluted and assayed.

Coleoptile section straight-growth assay. The technique used was identical with that described by Bentley and Housley (1954) and used in the work described in the first paper of this series (Phillips and Wareing, 1958). The sections were initially 10 mm. in length. Their final length, after 16–20 hours' growth at 26° C., was measured by means of a photographic enlarger. Ten replicates per treatment were used, and the mean final section length (in mm.) determined.

Lettuce seed germination assay. Approximately 80 seeds of the variety 'New Market' (a non-light-requiring type) were planted in each dish, and the percentage germination determined after 48 hours, at 22° C., in the dark.

Statistical analysis of results. Statistical anlyses of wheat coleoptile section assays were carried out by a 'short-cut' procedure using a method of allowances. This method of statistical analysis for analysing data which are usually handled by the methods of analysis of variance was prepared by Richard F. Link and David L. Wallace in connexion with research sponsored by the United States Office of Naval Research.

This method was developed to cover both one-way classification data (where there are several groups of measurements, each group being of the same size), and for two-way classification data (rectangular arrays of measurements, where both rows and columns are meaningful). Since the assays to be analysed fall into the first type of data (i.e. one-way classification), only the procedure to be adapted for such data was used.

Briefly, the method involves summing the ranges obtained from the individual treatments (10 replicates per treatment), and multiplying the total range so obtained by a factor from a table of critical factors for allowances. The resulting figure gave the difference between totals that was necessary at P 1 per cent. for significance. This difference figure was therefore divided by ten to give the difference needed between means for significance.

If an analysis of variance of the same data were performed, and the standard deviation derived, an allowance very little different from that obtained by the 'short-cut' method would have been secured.

EXPERIMENTAL

In Expts. 1, 3, and 4, photoperiodic treatments were used in which the supplementary illumination given in the L.D.-treated plants was provided by two 40-watt tungsten filament lamps (giving an intensity of about 500 lux). In Expt. 2, all the illumination, including both L.D. and S.D. treatments, came from 'daylight' fluorescent tubes (intensity = 8,000–10,000 lux) in a growth-cabinet.

Experiment 1

The treatments were commenced on 7 June. Sixty-seven plants were exposed to S.D., and 62 to L.D. Both S.D. and L.D. plants received 9 hours of high-intensity illumination (daylight) per cycle. The L.D. plants received a

further 9 hours' low-intensity illumination. The duration of the dark period was 15 hours for the S.D. plants and 6 hours for the L.D. plants.

The high-intensity illumination was given at greenhouse temperature, while the low-intensity illumination and dark periods were supplied in thermostatically controlled, electrically heated, light-proof cabinets at 70° F.

The treatments were ended on 26/6/56 (i.e. after 19 days). The S.D. plants had ceased growth by this time, as shown by the suppressed internodes in the apical region of the shoot. The apical regions and mature leaves were then removed for extraction.

The extracts of the shoot apices and mature leaves were chromatographed and assayed with both wheat coleoptile sections and lettuce seed. The chromatograms of the extracts of the shoot apices proved to be seriously overloaded, so that they 'trailed' badly, and the results are not presented here.

For the coleoptile section assay of the leaf extracts the volume of extract loaded in each case was equivalent to 2·5 g. fresh weight of tissue.

The results (Fig. 1) indicated that the pattern of activity on the chromatograms was similar to that observed in the previous investigation (Phillips and Wareing, 1958). This inhibition was evident in the region R_f 0–0·1, but for the reasons previously given (Phillips and Wareing, *loc. cit.*) no attention was paid to this region of inhibition. In addition, the other inhibitory zone occurring at approximately R_f 0·7 was also present in both extracts. It is seen that there is greater activity of this inhibitory zone in the extracts of the S.D. leaves than in the corresponding zone of the chromatogram of L.D. leaf extracts.

For the assay with lettuce seeds (Fig. 2), larger amounts of extracts (each equivalent to 6·0 g. fresh wt. of tissue) were chromatographed. Each of the chromatogram strips was eluted with 4·0 ml. of water. In this assay a most pronounced difference between the inhibitor contents of L.D.- and S.D.-treated plants was found. It is seen (Fig. 2A) that there is practically no inhibition of germination (except in the zone R_f 0·0–0·1) by the extract of leaves from the L.D. plants, but marked inhibition in the zone R_f 0·6–0·8 with the extract from leaves of plants grown under S.D. conditions (Fig. 2B).

Experiment 2

This was carried out as a repeat of the first experiment, in order to check the results obtained when the chromatograms of extracts of mature leaves were assayed, and to determine whether the chromatography of lower equivalent wts. of extracts of apices would give better separation of the inhibitor and other substances.

Short-days of 9 hours' light, 15 hours' dark, and L.D.s of 18 hours' light/6 hours' dark, were given at 15° C. in a growth-cabinet. Illumination was supplied by fluorescent tubes (intensity = 8,000–10,000 lux). These treatments were continued for 3 weeks, after which the apices and mature leaves

were harvested. Only leaves which had expanded during the treatment were taken for extraction, and these were usually the uppermost three pairs of fully expanded leaves.

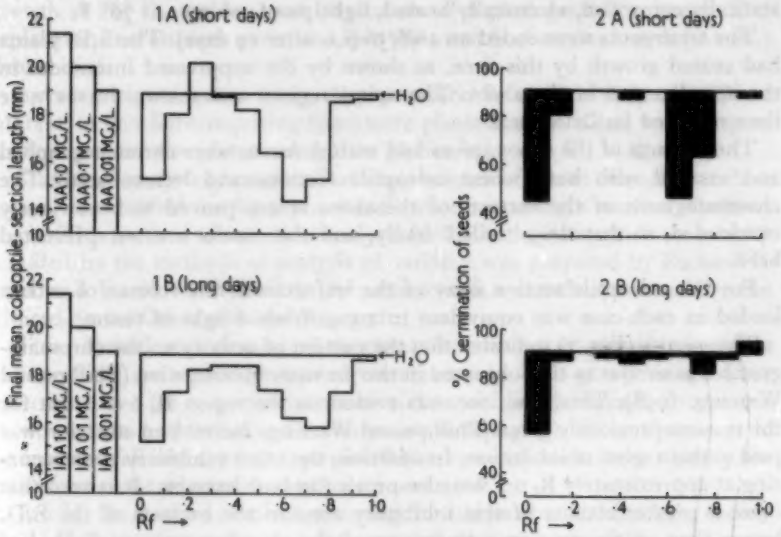


FIG. 1. Coleoptile section assay of chromatograms of extracts from mature leaves of plants grown under S.D.s (1A) and L.D.s (1B). Extract equivalent to 2·5 g. fresh wt. of tissue was chromatographed in each case. A difference of 1·55 mm. at $P<1$ per cent. between any two treatments, or between a treatment and control, is needed for statistical significance.

FIG. 2. Lettuce seed (var. 'New Market') germination assay of chromatographed extracts (each equivalent to 6·0 g. fresh wt. of tissue) of mature leaves. The one extract (2A) came from leaves of plants growing under L.D.s and the other (2B) from plants growing under S.D.s.

As before, the harvested tissues were frozen and extracted with 80 per cent. MeOH.

Extract equivalent to 0·105 g. dry wt. of each of the two samples of apices was chromatographed and assayed (Fig. 3). Inhibition was evident in regions R_f 0·5–0·8 of both chromatograms, and, as was observed in Expt. 1, there was more inhibitor present in the extract from S.D.-grown plants (Fig. 3A) than in that from L.D.-treated seedlings (Fig. 3B).

Similarly, when volumes, each equivalent to 0·51 g. dry wt., of the extracts of mature leaves were assayed (Fig. 4), more inhibitor was found to be present in the extract obtained from the leaves of the S.D.-grown plants (Fig. 4A) than in that from L.D.-treated plants. In fact, when smaller quantities (equivalent to 0·1 g. dry wt. of tissue) of extracts of leaves were chromatographed (Fig. 5) only in the extract prepared from plants grown under S.D.s (Fig. 5A) was inhibitor detectable in the wheat coleoptile section assay.

Thus in the first two experiments it was found that following approximately 3 weeks' growth under conditions of controlled daylength, the seedlings had developed differential levels of an inhibitor, both at the apex and in the mature leaves.

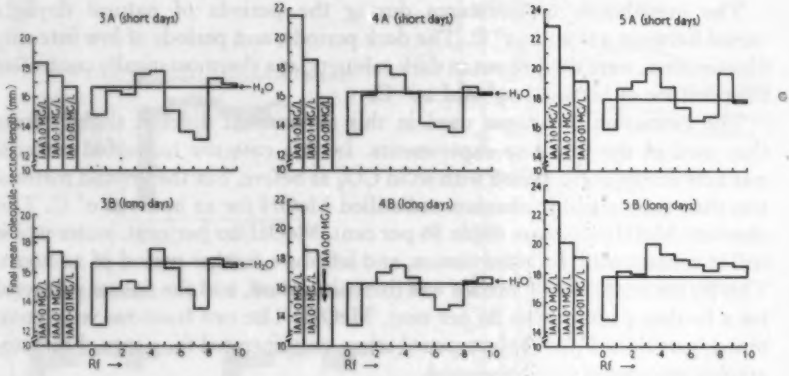


FIG. 3. Result of a coleoptile section assay of extracts of the apices of long- (3B) and short-day (3A) treated plants. Extract equivalent to 0·105 g. dry wt. was chromatographed in both cases. A difference of 1·10 mm. between any two treatments, or between a treatment and control, is needed for significance at $P<1$ per cent.

FIG. 4. Assay with coleoptile sections of extracts of mature leaves, after the equivalent of 0·51 g. dry wt. of tissue had been chromatographed. A difference of 1·37 mm. between any two treatments, or between a treatment and control, is needed for significance at $P<1$ per cent.

FIG. 5. Assay with wheat coleoptile sections of chromatographed extracts (each equivalent to 0·1 g. dry wt. of tissue) of mature leaves. A difference of 1.60 mm. between any two treatments, or between a treatment and control, is needed for significance at $P<1$ per cent.

However, the fact that S.D. treatment brings about both the cessation of growth by the shoot and an increase in the concentration of this inhibitor does not necessarily mean that the relationship between inhibitor and shoot growth is a causal one. This is especially true since the S.D.-treated plants had ceased growth by the time harvesting took place, whilst the L.D.-treated plants were still elongating. In other words it is possible that the increased inhibitor contents of S.D.-treated plants is actually the *result* of the cessation of growth rather than the *cause* of it. Because of this the following two experiments were performed in order to investigate how quickly a difference in inhibitor content between L.D.- and S.D.-treated plants becomes apparent.

Experiment 3. Effects of various numbers of either S.D. or L.D. cycles upon the natural growth inhibitor content of seedlings

The seedlings used in this and the next experiment were grown in a heated greenhouse until 20 May when they were transferred to cold frames. They were brought back into the greenhouse on 30 May and the photoperiodic

treatments were commenced on that date. A total of 263 plants was used for this and the fourth experiments.

The S.D.s consisted of 9 hours natural daylight and 15 hours darkness, the L.D.s of 9 hours natural daylight supplemented by 9 hours of low intensity illumination+6 hours darkness.

The greenhouse temperatures during the periods of natural daylight varied between 21° and 32° C. The dark periods, and periods of low intensity illumination, were carried out in dark cabinets, at a thermostatically controlled temperature of between 19° and 20° C.

The extraction technique used in this experiment differed slightly from that used in the first two experiments. In this case the harvested material was first frozen and crushed with solid CO_2 as before, but the ground material was then submerged in absolute redistilled MeOH for 22 hours at 0° C. This absolute MeOH was then made 80 per cent. MeOH/20 per cent. water whilst still in contact with the plant tissues, and left for a further period of 22 hours. This 80 per cent. MeOH extract was then filtered off, and the tissues extracted for a further 4 hours with 80 per cent. MeOH. The two fractions were combined, partitioned with 80/100 petrol ether, concentrated for paper chromatographic separation and bioassayed.

Samples of apices and mature leaves were taken after exposure of the plants to 2, 5, 10, and 33 cycles of either S.D. or L.D. Samples were also taken of both apices and mature leaves immediately before the commencement of the photoperiodic treatments.

The result of the simultaneous assay (wheat coleoptile sections) of the extracts of the apices (each the equivalent of 0·17 g. dry wt. of tissue) is shown in Fig. 6.

The results show quite clearly that there is consistently more inhibitor in the extracts obtained from S.D.-treated plants than in extracts from plants grown under L.D. This is seen even after only 2 cycles of the photoperiodic treatments (Figs. 6B and 6C), though the difference is much more pronounced after 5 cycles (Figs. 6D and 6E). After this time the inhibitor content of the apices of L.D.-treated plants increased, though it never reached the same level as that of the S.D.-treated plants.

The inhibitor content of the apices of L.D.-treated plants apparently decreased between the start of the experiment (Fig. 6A) and 5 cycles after the start (Fig. 6D). The inhibition produced by the extract of apices of the plants exposed to S.D. (Fig. 6C) is also slightly less than that in Fig. 6A. The apparent decrease in inhibitor, both under L.D. and S.D. conditions, is quite possibly due to the transfer of plants, at the beginning of the experiment, from cold frames into a heated greenhouse. That is, the increase in temperature may have had the effect of causing the decrease in the inhibitor concentration.

The assay of the extracts of the mature leaves made in this experiment were carried out as follows.

The equivalent of 1·0 g. dry wt. of each extract was chromatographed on a paper 11·6 cm. in width, and the zone R_f 0·55–0·88 was cut out and eluted

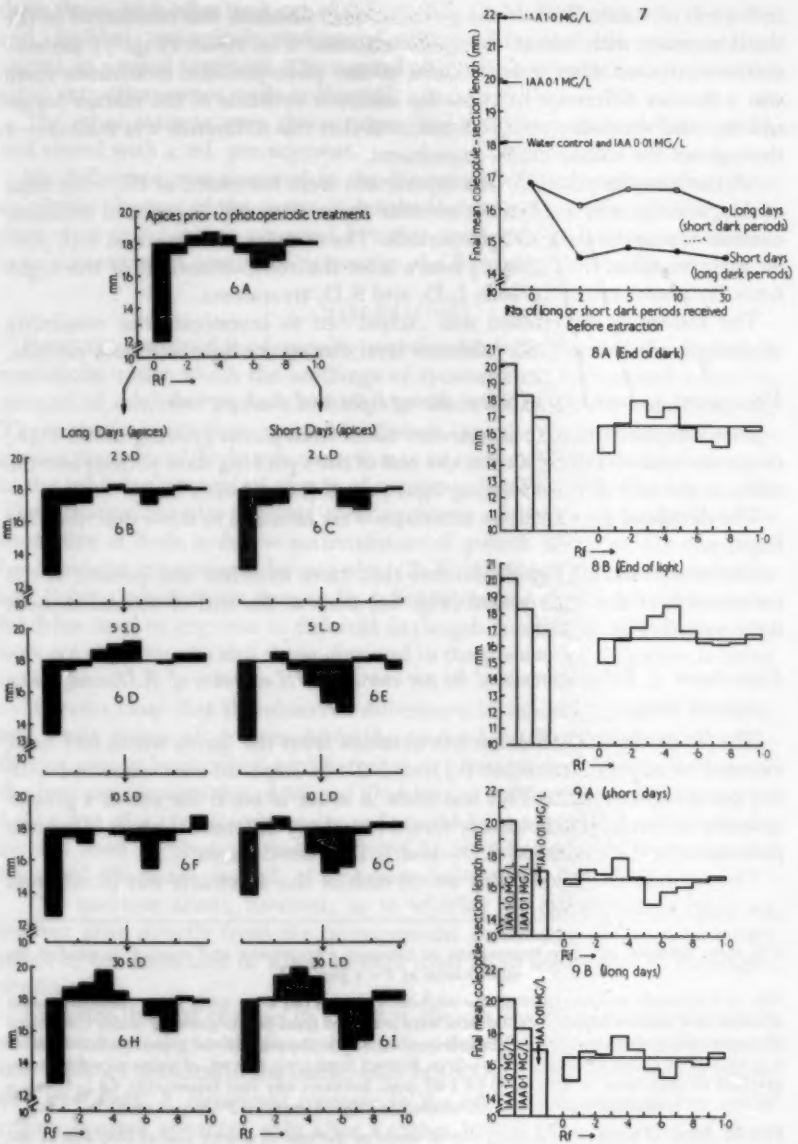


FIG. 6. Simultaneous assay of chromatographed extracts of apices of photoperiodically treated plants with coleoptile sections. A volume of extract equivalent to 0·17 g. dry wt. of tissue was assayed in each case. The extracts were prepared after exposure of the plants to 2, 5, 10, and 30 cycles of the photoperiodic treatment (either short-days or long-days). A sample was also taken immediately before the start of the treatments (6A). A difference of

in 8·0 ml. of water. Each of the 9 eluates thus obtained was bioassayed in the usual manner with wheat coleoptile sections. The result (Fig. 7) demonstrates that even after only 2 cycles of the photoperiodic treatments there was a distinct difference between the inhibitor contents of the mature leaves of long- and short-day grown plants, and that this difference was maintained throughout the course of the experiment.

All the samples taken in this experiment were harvested at the same time of day, since it was not known whether there were any changes in inhibitor content during the dark or light periods. The samples were taken at 2.30 p.m. on each occasion, this being 6½ hours after the commencement of the high-intensity photoperiod for both L.D. and S.D. treatments.

The following experiment was carried out to investigate the possibility of changes occurring in the inhibitor level during the light and dark periods.

Experiment 4. Levels of inhibitor during light and dark periods

Two samples of mature leaves were taken from plants growing under S.D., one at the end of 13th cycle (i.e. the end of the 13th long dark period) and the other at the end of the following light period (i.e. 9 hours later).

The details of the extraction technique were identical to those described for Expt. 3.

The assay results (Fig. 8) indicate that more inhibitor was present at the termination of the dark period (Fig. 8A) than at the end of the subsequent light period (Fig. 8B).

Experiment 5. Ether extracts of 80 per cent. MeOH extracts of S.D. and L.D.-exposed leaves

The 80 per cent MeOH extracts obtained from the leaves which had been exposed to 10 cycles of either S.D. or L.D. in Expt. 20 were extracted with dry peroxide-free ether. This was done in order to see if the use of a greater quantity of material in a bioassay would reveal any differential levels of growth promoters in the extract of S.D.- and L.D.-treated leaves.

The equivalent of 8·0 g. dry wt. of each of the 2 extracts was partitioned

---

1·12 mm. between any two treatments, or between a treatment and control, is needed for significance at $P<1$ per cent.

FIG. 7. Coleoptile section assay of the inhibitory eluates (R_f 0·55–0·88) from chromatograms of extracts of mature leaves. The extracts were prepared from plants growing under controlled photoperiodic conditions; the leaf samples were made at the start of the experiment, and after 2, 5, 10, and 30 cycles of long- or short-days. Extract from 1·0 g. dry wt. of tissue was chromatographed in each case. A difference of 1·08 mm. between any two treatments, or between a treatment and control, is needed for significance at $P<1$ per cent.

FIG. 8. Assay of extracts of mature leaves made at the end of a dark period (8A) and at the end of the subsequent light period (8B). Each extract was equivalent to 0·5 g. dry wt. of tissue. A difference of 1·27 mm. between any two treatments, or between a treatment and control, is needed for significance at $P<1$ per cent.

FIG. 9 Coleoptile section assay of ether extracts of 80 per cent. methanol extracts of mature leaves. 9A, from plants growing under short-days; 9B, from plants under long-days.

three times with ether (volume of S.D. and L.D. MeOH extracts=12·85 ml. and 12·58 ml. respectively; volume of ether used for each extraction totalled 100 ml. in 3 equal portions). The natural pH of the 2 extracts was 3·2, and the ether extractions were made at this pH.

The ether extracts were chromatographed on papers each 11·6 cm. wide, and eluted with 4 ml. per segment.

No difference was observed in the histograms of the result of the wheat coleoptile bioassay of the chromatographed ether extracts (Fig. 9). Only faint promotion or inhibition occurred in either case, and it would appear that no large quantities of auxin are present in the 80 per cent. MeOH extracts.

DISCUSSION

From the results of the foregoing experiments it is clear that the daylength conditions under which the seedlings of sycamore are maintained affect the amount of inhibitor present in both the shoot apex and the mature leaves. These results, with those quoted by Nitsch (1957), give support to the suggestion that natural daylength changes may be responsible for seasonal changes in the inhibitor content of shoots of sycamore (Phillips and Wareing, 1958). The observations also support the hypothesis of Hemberg (1949) that the dormancy of buds is due to accumulation of growth inhibitor. On the other hand, similar experiments by one of us (P. F. Wareing) with *Betula pubescens* and *Robinia pseudoacacia* have so far failed to reveal a significant difference in inhibitor level in response to different daylength conditions. The discrepancy between these results and those obtained in the present investigation is being investigated.

It seems clear that the observed differences in inhibitor content in leaves and shoot apices of *A. pseudoplatinus* under long-day and short-day conditions cannot be ascribed to differences in photosynthesis, since in three of the four experiments the additional illumination received by the plants under L.D.s was of a very low intensity and would have a negligible effect on the general level of photosynthetic activity. It would seem, therefore, that the observed effects are, indeed, of a photoperiodic nature.

The question arises, however, as to whether the differences in inhibitor content arise *directly* from the photoperiodic conditions, or are a *secondary* effect of the reduction in apical growth itself from differences in daylength conditions.

Evidence that the changes in inhibitor content are not primarily the result of reduced apical growth is seen by the fact that an increase in inhibitor content could be detected in the *leaves*, after exposure to only *two* cycles of short-day treatment. A significant increase in the inhibitor content of the *apical* region became apparent only after a rather longer time (5 cycles of short days). This sequence of changes is compatible with the hypothesis that the inhibitor is produced in the leaves and transported to the shoot-apex.

It has been seen that the differences in inhibitor content as between seedlings grown under L.D. and those maintained under S.D. is of a 'quantitative'

nature, and that inhibition is present even in shoot apices growing under long-day conditions. If the cessation of growth which occurs when the seedlings are transferred to S.D. results from the increased inhibitor production under the latter conditions, then it would appear that the behaviour of the shoot apex is determined by whether the inhibitor content lies above or below a certain 'threshold' value.

It is possible that the response of the shoot apex of sycamore to photoperiod is controlled by an interaction between growth promoters and growth inhibitors. A growth-promoting region was present on most chromatograms between R_f 0·2 and 0·5, but no attempt was made to relate the activity of this region with the photoperiodic conditions, since there is evidence that the promotion in this region is due to the presence of sugars rather than growth hormones (Phillips and Wareing, 1958). The amount of 80 per cent MeOH extract assayed with wheat coleoptile sections never exceeded that from 1·0 g. dry wt. of tissue, from which only very small amounts of auxins could expect to be obtained. It is possible, however, that extractions of larger amounts of material might reveal differences in auxin level under L.D. and S.D. conditions, though in Expt. 5 the equivalent of 8·0 g. dry wt. was assayed, and it might be expected to obtain auxin activity from this quantity of material. Several earlier workers reported greater production of auxin under L.D. than under S.D. conditions (e.g. Leopold, 1949), but it has proved difficult to correlate these differences with the observed photoperiodic responses in relation to flowering.

LITERATURE CITED

Bentley, J. A., and Housley, S. (1954). Bioassay of plant growth hormones. *Physiol. Plantarum*, **7**, 405.

Hemberg, T. (1949). Growth-inhibiting substances in terminal buds of *Fraxinus*. Ibid. **2**, 37.

Leopold, A. C. (1949). The control of tillering in grasses by auxin. *Amer. J. Bot.* **36**, 437.

Nitsch, J. P. (1957). Growth responses of woody plants to photoperiodic stimuli. *Proc. Amer. Soc. Hort. Sci.* **70**, 512.

Phillips, I. D. J., and Wareing, P. F. (1958). Studies in dormancy of sycamore. I. Seasonal changes in the growth-substance content of the shoot. *J. Exp. Bot.* **9**, 27, 350.

Wareing, P. F. (1951). Growth studies in woody species. III. Further photoperiodic effects in *Pinus sylvestris*. *Physiol Plantarum*, **4**, 41.

—— (1954). Growth studies in woody species. VI. The locus of photoperiodic perception in relation to dormancy. Ibid. **7**, 261.

ERRATUM

Vol. 10, No. 28, p. 91, line 5 from the foot should read:

If X is reduced to half, this term still is less than 0·4 . . .

continued from p. ii of cover]

Correspondence should be addressed as follows:

On editorial matters to Professor W. T. Williams, Department of Botany, University of Southampton, England.

With regard to membership of the Society for Experimental Biology, to the Hon. Botanical Secretary, Dr. G. E. Fogg, Department of Botany, University College, Gower Street, London, W.C. 1.

Members of the Society not yet receiving the Journal regularly who wish to become subscribers are asked to communicate with the Publications Secretary, c/o The Institute of Biology, 41 Queen's Gate, London, S.W. 7, who will deal with all matters of supply of the Society's journals to members.

On all other matters, to the publishers, Oxford University Press, Amen House, Warwick Square, London, E.C. 4.

BOOKS

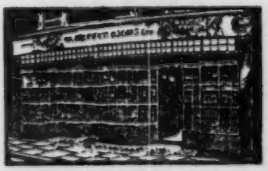
A Large Stock of Recent Editions on BOTANY & ALLIED SCIENCES.
Catalogue sent on request.

SECONDHAND DEPT.
A select stock of recent editions available. Back numbers of scientific journals obtainable.

LENDING LIBRARY
Scientific and Technical
Annual Subscription £1. 17s. 6d.
Prospectus post free on request.
THE LIBRARY CATALOGUE revised to December 1956. To Subscribers, £1. 5s.; to Non-Subscribers, £2. 2s. Postage, 2s.

H. K. LEWIS & Co. Ltd.
136 GOWER STREET, W.C.1
EUSton 4282

HEFFER'S



BOOKS ON BOTANY IN ALL LANGUAGES

★

Catalogues available free; books and learned journals bought

★

W. HEFFER & SONS LTD.
Petty Cury · Cambridge

complete

High-speed and Super-speed Centrifuges

HIGH-SPEED 10
without refrigeration

Max. speed 10,000 r.p.m. ● Centrifugal force 10,000 x g. ● 100 ml. maximum tube size ● 400 ml. maximum total capacity.

HIGH-SPEED 17
with or without refrigeration

Max. speed 17,000 r.p.m. ● Centrifugal force 35,000 x g. ● 250 ml. maximum container size ● 1,500 ml. maximum total capacity.

SUPER-SPEED 25
with refrigeration

Max. speed 25,000 r.p.m. ● Centrifugal force 75,000 x g. ● 50 ml. maximum tube size ● 400 ml. maximum total capacity.

MSE

MEASURING & SCIENTIFIC EQUIPMENT LTD.
Spenser Street, London, S.W.1. Tel.: VICtoria 5426

MICHROME STAINS
and Biological Reagents

Adenosine	Cytase	Methyl Green
Adenylic Acids	Fast Green	Pyronin
Carminic Acid	Haematoxylin	Ribonuclease
Cedarwood Oil	Janus Green	Sudan Black
Celloidin	Leishman Stain	Toluidine Blue
Cellosolve	Lignin Pink	Urease
Chlorazol Black	Lipase	&c.

AQUAMOUNT: synthetic, neutral, colourless aqueous mountant

62-page Catalogue available on request

Ready January 1960
ENCYCLOPAEDIA OF MICROSCOPIC STAINS by Edward Gurr, about 300 pages, Royal 8vo, about 95s.

EDWARD GURR, LTD.
42 Upper Richmond Road West
LONDON, S.W. 14
Telephones: PROspect 8051 and 7606
Service, uniform high quality, reliability, and immediate delivery

Now ready:
MICROSCOPIC STAINING TECHNIQUES, No. 4. (1st edit.) 66 pages. Price 6s. ($1.00 in U.S.A.) No. 3. (2nd edit.) 66 pages. Price 6s. ($1.00 in U.S.A.)
'METHODS OF ANALYTICAL HISTOLOGY AND HISTOCHEMISTRY,' by Edward Gurr. First edition. Royal 8vo, 334 pages. 70s. net.

BOTANICAL BOOKS

New, Secondhand
Old and Rare

WHELDON & WESLEY LTD.
LYTTON LODGE
Codicote, Near Hitchin
Herts.
Telephone: CODICOTE 370

Libraries and small parcels of books purchased for cash

Printed in Great Britain at the University Press, Oxford, by Vivian Ridler, Printer to the University

189